CIBELLY GOULART

**VACINAS PNEUMOCÓCICAS PROTEICAS,
AVALIAÇÃO DA RESPOSTA IMUNE SOB
DIFERENTES APRESENTAÇÕES**

Tese apresentada ao Programa de Pós-Graduação Interunidades em Biotecnologia USP/Instituto Butantan/IPT, para obtenção do Título de Doutor em Biotecnologia.

São Paulo
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Orientadora: Luciana Cezar de Cerqueira Leite

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CERTIFICADO

Certificamos que o Projeto intitulado "Vacinas anti-pneumocócicas baseadas em PspA, PdB e polissacarídeos: avaliação da reatividade cruzada e mecanismo de resposta imunológica", protocolo nº 602/09, sob a responsabilidade de Luciana C. C. Leite e Cibelly Goulart, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO INSTITUTO BUTANTAN (CEUAIB) em reunião de **15/04/2009**.

We certify that the research entitled "Anti-pneumococcal vaccines based on PspA, PdT and PS: cross-reactivity and immune response", protocol nº 602/09, under the responsibility of Luciana C. C. Leite and Cibelly Goulart, is in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation, and was approved by the ETHICAL COMMITTEE FOR ANIMAL RESEARCH of BUTANTAN INSTITUTE in the meeting of 04/15/2009

Vigência do Projeto: 04/2009 – 02/2014 Centro de Biotecnologia	Nº de animais/espécie 960/ camundongos Balb/c fêmeas
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São Paulo, 15 de Abril de 2009.

Dra. Denise V. Tambourgi
Presidente da CEUAIB

De acordo:

Dr. Otávio Azevedo Mercadante
Diretor do Instituto Butantan

*À Família e amigos, que foram meu alicerce
durante essa jornada*

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"Se eu gosto de poesia? - Gosto de gente, bichos, plantas, lugares, chocolate, vinho, papos amenos, amizade, Amor. Acho que a poesia está contida nisso tudo."

- Carlos Drummond de Andrade

"Tive um verdadeiro cansaço em Paris de gente inteligente. Não se pode ir a um teatro sem precisar dizer se gostou ou não, e por que sim e por que não. Aprendi a dizer "não sei", o que é um orgulho, uma defesa e um mau hábito porque termina-se mesmo não querendo pensar, além de não querendo dizer. (Cartas perto do Coração - a Fernando Sabino – 8/2/1947)."

- Clarice Lispector

"A questão que às vezes me deixa louco; Louco sou eu ou são os outros?"

- Albert Einstein

RESUMO

GOULART, C. **Vacinas pneumocócicas proteicas, avaliação da resposta imune sob diferentes apresentações.** 2014. 178 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014

Doenças pneumocócicas constituem um importante problema de saúde pública. Diferentes proteínas pneumocócicas têm sido estudadas como alternativas para vacinas. PspA é uma proteína exposta na superfície do pneumococo, expressa virtualmente em todas as cepas. Ply é uma citolisina dependente de colesterol. Ambas atuam como mecanismo de evasão do pneumococo e são capazes de induzir anticorpos essenciais para a proteção contra a sepse. As proteínas SP 0148 e SP 2108, uma transportadora ABC e outra ligante de maltose, foram descritas como potenciais candidatos vacinais por induzirem a produção de IL-17 e protegerem camundongos contra a colonização. Este trabalho teve como objetivo principal desenvolver vacinas pneumocócicas baseadas em proteínas. Na primeira etapa selecionamos uma molécula de PspA capaz de induzir uma ampla reatividade cruzada dentro da família 1. Em seguida, foi produzida uma proteína híbrida contendo a molécula de PspA, selecionada, fusionada ao PdT, um pneumolisóide derivado da Ply. A imunização de camundongos com PspA2-PdT promoveu a produção de anticorpos contra ambas as proteínas, com elevada afinidade a bactérias contendo PspAs heterólogas, e manteve a atividade opsonizante e anti-hemolítica destes. Além disso, essa imunização induziu a produção de citocinas inflamatórias e proteção após desafio letal. O BCG possui propriedades adjuvantes, assim, foram desenvolvidas vacinas baseadas em BCG recombinantes expressando as proteínas pneumocócicas rPspA-PdT, SP 0148 e SP 2108. Após a imunização de camundongos utilizando a estratégia de *prime/boost*, o rBCG-0148/rSP 0148 induziu a produção de IL-17 e IFN- γ em cultura de esplenócitos. A combinação das três vacinas de rBCG mostrou-se mais eficiente na proteção contra desafio de colonização. Em conjunto, esses dados sugerem que a apresentação da PspA e PdT em forma de proteína de fusão seria um potente candidato vacinal, capaz de aumentar a reatividade cruzada entre as diferentes cepas pneumocócicas. Além disso, a imunização com rBCG mostrou-se eficiente na indução da resposta imune específica, sendo capaz de reduzir o número de doses necessárias para induzir proteção contra pneumococo, sendo portanto, seu uso, promissor como vacina pneumocócica.

Palavras-chave: *S. pneumococo*. Complemento. Opsonização. PspA. PdT. rBCG. SP 0148. SP 2108.

ABSTRACT

GOULART, C. **Pneumococcal protein vaccines, evaluation of immune responses under different presentations.** 2014. 178 p. Ph. D. thesis (Biotechnology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014.

Pneumococcal diseases are a major public health problem. Several alternative vaccines have been proposed using more conserved pneumococcal proteins, as PspA and Ply. PspA is a surface-exposed protein, virtually expressed by all pneumococcal strains and Ply is a cholesterol-dependent cytolysin with many biologic functions. Both are required for full pneumococcal virulence and are able to induce protective antibodies against pneumococci. Other proteins, as SP 0148 and SP 2108, an ABC transporter and a Maltose binding protein, are able to induce IL-17 production and to protect mice against pneumococcal colonization. The major aim of this study was to produce pneumococcal vaccines based on proteins. First, we selected one PspA molecule able to induce broad-ranging cross-reactivity within family 1. Then, we constructed a hybrid protein containing a PspA fused to PdT, a detoxified form of Ply. The hybrid protein was able to induce antibodies against both proteins, with opsonic and anticytolytic activity and high affinity to heterologous PspAs. Furthermore, this protein was able to induce production of inflammatory cytokines and protect mice against lethal challenge. At last, due the adjuvant properties of BCG, we constructed recombinant BCG strains expressing the hybrid protein PspA-PdT, SP 0148 and SP 2108. Mice immunization with a prime-boost scheme showed that rBCG-0148/SP0148 induced production of IL-17 and IFN- γ in spleen cells culture. The combination of all rBCG vaccines was more efficient in protecting mice against pneumococcal colonization. These results suggest that presentation of PspA and PdT as a fusion protein is a potential vaccine, able to increase the cross-reactivity among different pneumococcal strains. Furthermore, the improvement of specific immune responses by rBCG immunization is able to reduce the number of doses required to induce protection against pneumococcus, suggesting its promising use as pneumococcal vaccine.

Keywords: *S. pneumoniae*. Pneumococcal vaccines. Complement. Opsonization. rBCG. PspA. PdT. SP 0148 and SP 2108.

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LISTA DE SIGLAS

μF, Micro farad

2YT, meio de cultura 2 x extrato de levedura e triptona

Al(OH)₃, Hidróxido de alumínio

BCG, Bacilo de Calmette e Guérin

BSA, albumina do soro bovino

C1q, proteína C1q do sistema complemento

C3, proteína C3 do sistema complemento

CBA, do inglês “*cytometric beads array*”

CDR, Região definidora de clados

CEUA/IB, Comissão de Ética no Uso de Animais/ Instituto Butantan

Chop, fosforilcolina

ConA, Concanavalina A

DMEM/F12, meio Eagle Modificado por Dulbecco + Nutriente HAM F-12

DNA, ácido desoxirribonucleico

dPLY, pneumolisóide detoxifica por formaldeído

E. coli, *Escherichia coli*

ELISA, ensaio imunoenzimático

EPEC, *E. coli* enteropatogênicas

FITC, Isotiocianato de fluoresceína

FMUSP, Faculdade de Medicina da Universidade de São Paulo

GAVI, Aliança Mundial para Vacinas e Imunização

HBSS, solução-tampão salina de Hank

HIV, vírus da imunodeficiência humana

HRP, peroxidase de raiz forte

IFN-γ, Interferon-gama

IgA, Imunoglobulina A

IgG, Imunoglobulina G

IL, Interleucina

IPTG, isopropil-β-D-1-tiogalactopiranosídeo

kDa, kilodaltons

kV, kilovolts

LB, meio Luria-Bertani

MB7H0, meio de cultura Middlebrook 7H10 glicerol 0,5%

MB7H9, meio de cultura Middlebrook 7H9 glicerol 0,5% e Tween 80 0,05%

MPLA, monofosforil lipídeo A

NaCl, cloreto de sódio

NanA, neuraminidase A

NanB, neuraminidase B

NF- κ B, factor nuclear kappa B

NLRP3, Receptor do tipo NOD 3

NMS, soro normal de camundongo

NON-PRO, bloco sem a presença do aminoácido prolina

OADC, suplemento do meio Middlebrook (ácido oleico-albumina-dextrose-catalase)

OPA, ensaio de morte opsonofagocitose

OPD, orto-fenilendiamina dicloridrato

PAF, fator ativador de plaquetas

PAFr, receptor fator ativador de plaquetas

PBS, solução salina tamponada

PcpA, proteína ligante de colina A

PCR, reação em cadeia da polimerase

PCV10, vacina pneumocócica 10-valente

PdT, pneumolisóide com 3 mutações

PhtD, histidine triad protein

Ply, pneumolisina

PIYD1, pneumolisóide detoxificado por mutagênese

PS, polissacarídeo

PspA, proteína de superfície de pneumococo A

PspC, proteína de superfície de pneumococo C

PT, toxina de *Bordetella pertussis*

PVDF, polivinil-difluorado

rBCG, BCG recombinante

RPMI, meio de cultura desenvolvido no Roswell Park Memorial Institute

SBF, soro bovino fetal

SDS-PAGE, eletroforese em gel de poliacrilamida e dodecil sulfato de sódio

TBS-T, tampão Tris salino + 0,5% Tween-20

Th1, Th2, Th17, Resposta auxiliar do tipo 1, 2 e 17

TLR4, Receptor do tipo Toll 4

TNF- α , Fator de necrose tumoral alfa

UFC, unidade formadora de colônia

UH, unidade hemolítica

Ω , ohms

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1 INTRODUÇÃO

Doenças pneumocócicas representam um importante problema de saúde pública, afetando cerca de 14 milhões de pessoas anualmente (O'BRIEN et al., 2009). A pneumonia é a principal causa de mortalidade infantil, sendo responsável por cerca de 18% do total de mortes anuais (LIU et al., 2012). As doenças pneumocócicas afetam principalmente os países em desenvolvimento, particularmente na África e Ásia (Figura 1) e causam aproximadamente 500 mil mortes em crianças menores de 5 anos, excluindo-se os casos de coinfeção por HIV. Além disso, doenças pneumocócicas, possuem elevada incidência em adultos acima de 65 anos e indivíduos imunodeprimidos (O'BRIEN et al., 2009; WORLD HEALTH ORGANIZATION, 2012)

Figura 1. Incidência de doenças pneumocócicas por 100.000 crianças abaixo de 5 anos. (WORLD HEALTH ORGANIZATION, 2009).

1.1 *Streptococcus pneumoniae* – da colonização à infecção

Streptococcus pneumoniae é uma bactéria Gram positiva, encapsulada que coloniza as vias aéreas de indivíduos saudáveis - principalmente crianças - e pode causar doenças como otite média, pneumonia, meningite, bacteremia, entre outras (HENRIQUES-NORMARK, TUOMANE, 2013). *In vitro*, pneumococos podem ser cultivados em ágar sangue, onde apresentam colônias α -hemolíticas, sensíveis à optoquina, e solúveis em sais biliares (CHANDLER et al., 2000).

A superfície da bactéria que é constituída de 3 estruturas - membrana plasmática, parede celular e cápsula polissacarídica, e ancora diversos fatores de virulência como mostrado na Figura 2 (DOWSON, 2004; KADIOGLU et al., 2008).

Figura 2. Esquema de um pneumococo mostrando a localização de alguns de seus importantes fatores de virulência (KADIOGLU et al., 2008)

A cápsula polissacarídica é o principal fator de virulência da bactéria, protegendo-a da fagocitose pelo sistema imune, da deposição de componentes do sistema complemento e anticorpos (ABEYTA; HARDY; YOTHER, 2003; BOGAERT; DE GROOT; HERMANS, 2004; WINKELSTEIN; ABRAMOVITZ; TOMASZ, 1980). Com base em diferenças na composição e estrutura química dos políssacarídeos que formam a cápsula, os pneumococos foram classificados em cerca de 93 sorotipos, constituindo 48 grupos sem reatividade cruzada (CALIX; NAHM, 2010; YOTHER, 2004). A maior parte das doenças pneumocócicas está associada a um número restrito de sorotipos, sendo que 6-11 sorotipos são responsáveis por aproximadamente 70% dos casos (JOHNSON et al., 2010). Outros fatores de virulência incluem: i) as Proteínas de superfície de pneumococo A e C, que se ancoram em resíduos de fosforilcolina (Chop) presentes no ácido teicóico da parede celular e também nos resíduos de ácido lipoteicóico que estão associados à bicamada lipídica da membrana celular (BROOKS-WALTER; BRILES; HOLLINGSHEAD, 1999; HOLLINGSHEAD; BECKER; BRILES,

2000); ii) proteínas ligantes de metal como antígeno de superfície de pneumococo A (TSENG et al., 2002); iii) proteínas de aquisição e transporte de ferro PiaA e PiuA (BROWN et al., 2001); iv) pneumolisina, uma citolisina dependente de colesterol; V) autolisina A, que promove a lise da parede do pneumococo provocando a liberação da pneumolisina e do ácido teicóico – conhecido como polissacarídeo C, ambos responsáveis pela resposta inflamatória intensa observada na infecção por pneumococo, entre outros (MARTNER et al., 2009; PATON; FERRANTE, 1983).

A colonização da nasofaringe inicia-se logo após o nascimento atingindo cerca de 40-60% das crianças menores de 5 anos; após essa idade a colonização é reduzida e em adultos ocorre em cerca de 3-4% dos indivíduos (CARDOZO et al., 2008; DAGAN et al., 1996). A colonização da nasofaringe é o primeiro passo para o estabelecimento de doenças pneumocócicas, sendo também essencial para a transmissão do pneumococo, que é eliminado juntamente com gotículas da respiração. Entretanto, indivíduos saudáveis podem ser colonizados sem que desenvolvam qualquer doença. Fatores de risco como idade inferior a 2 anos, má nutrição, coinfeção por vírus da influenza A, imunodeficiências, doenças cardíacas, diabetes e tabagismo (ativo ou passivo), estão associados ao desenvolvimento das doenças (MEHR; WOOD, 2012). O processo de colonização da nasofaringe exige a participação de diversos fatores de virulência, conforme resumido na Figura 3, e envolve a clivagem do muco por exoglicosidases (BURNAUGH; FRANTZ; KING, 2008; KING; HIPPE; WEISER, 2006), a atividade da IgA protease (WEISER et al., 2003), a redução dos batimentos ciliares induzida pela pneumolisina (FELDMAN et al., 1990; FELDMAN et al., 2002) e proteção contra a ação bactericida da lisozima e lactoferrina (DAVIS et al., 2008; SHAPER et al., 2004; VOLLMER; TOMASZ, 2002). A adesão do pneumococo ocorre por interação com glicoconjugados da superfície do epitélio e envolve produtos expressos pelos genes *spxB*, *ami*, *msrA* e *plpA* (CUNDELL et al., 1995a; SPELLERBERG et al., 1996; WIZEMANN et al., 1996). Ocorre também a interação entre PspC e o receptor do fator ativador de plaquetas (PAF) e de Chop com o receptor polirico de imunoglobulinas (pIg) (CUNDELL et al., 1995b; ELM et al., 2004; KAETZEL, 2001). A colonização pode induzir uma resposta imunológica que leva à eliminação da bactéria. Doenças pneumocócicas (Figura 4) podem ocorrer pelo espalhamento do pneumococo na cavidade nasal, levando ao desenvolvimento de sinusites e otites médias ou pela aspiração da bactéria para o pulmão, onde pode levar à inflamação e pneumonia. Doenças invasivas podem ocorrer pela penetração do pneumococo em lesões do pulmão, pela translocação bacteriana mediadas pelos receptores PAF e pIg – que podem facilitar a passagem do pneumococo para a corrente sanguínea (CUNDELL et al., 1995b; KAETZEL, 2001;

ZHANG et al., 2000) ou ainda pela migração através dos espaços inter ou pericelulares da camada celular do epitélio, através de interações com plasminogênio - que envolvem a ligação de plasmina e clivagem de proteínas das junções celulares, em especial a caderina (ATTALI et al., 2008a; ATTALI et al., 2008b; PANCHOLI; FONTAN; JIN, 2003). As meningites podem ocorrer pela passagem do pneumococo da corrente sanguínea para as meninges ou então diretamente da nasofaringe para o cérebro através do bulbo olfatório (VAN GINKEL et al., 2003).

Figura 3. Colonização e invasão do hospedeiro pelo pneumococo. A) A colonização da nasofaringe por pneumococos é facilitada pela degradação do muco pelas enzimas NanA, NanB, BgaA, StrH e também pela Ply que reduz o batimento ciliar, favorecendo a aderência da bactéria. B) PdgA e Adr protegem a parede de peptoglicano do pneumococo da degradação por lisozima, enquanto sIgA é clivada pela IgA protease bacteriana. C) *S. pneumoniae* liga-se a GalNac presente nas células epiteliais do hospedeiro através de moléculas de SpxB, Smi, MsrA, and PlpA. D) Pneumococo se liga a células do hospedeiro por interação das proteínas ChoP e PspC com receptores PAFr e PlgR, promovendo a translocação da bactéria. E) Migração inter e pericelular, mediada pela interação de proteínas do pneumococo com plasminogênio (MOOK-KANAMORI et al., 2011).

1.1.1 Vacinas Polissacarídicas contra *S. pneumoniae*

Os polissacarídeos capsulares constituem a base das formulações vacinais atualmente em uso contra infecções pneumocócicas, sendo administrados em sua forma livre ou conjugados a proteínas carreadoras. A primeira vacina polissacarídica contra *S. pneumoniae* foi produzida em 1977, e era composta de PS dos 14 sorotipos prevalentes na Europa e Estados Unidos (AUSTRIAN; GOLD, 1964). Esta foi substituída em 1983 por uma formulação 23-valente, utilizada até os dias atuais sob os nomes de Pneumovax 23 (Merck Research Laboratories, EUA) e Pneumo 23 (Sanofi-Pasteur, França). Estas contêm os PS dos sorotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F e

33F, apresentando cobertura de até 90% contra pneumonia em adultos jovens. No entanto, a imunização com PS induz uma resposta imunológica do tipo T-independente, ineficaz em crianças abaixo dos 2 anos e indivíduos imunodeprimidos, que compreendem dois dos grupos de maior risco. (KOSKELA et al., 1986; LEINONEN et al., 1986; O'BRIEN et al., 1996). Em idosos, a eficácia desta formulação contra pneumonia é reduzida, não ultrapassando 60%. Além disso, vacinas polissacarídicas não induzem memória imunológica, sendo necessária a revacinação a cada cinco anos. Outro problema é que esta vacina não induz proteção contra otite média, uma das consequências mais comuns da infecção por pneumococo (WADWA; FEIGIN, 1999). Finalmente, muitos PS são pouco imunogênicos, incluindo aqueles associados com resistência a antibióticos (POLAND, 1999).

Figura 4. Rota da patogênese por pneumococo (adaptado (BOGAERT; DE GROOT; HERMANS, 2004).

1.2 Profilaxia – Vacinas pneumocócicas

1.2.1 Vacinas Conjugadas

Polissacarídeos estimulam a produção de anticorpos sem auxílio de células T, sendo denominados antígenos T-independentes, por esta razão, falham na indução de uma resposta imunológica efetora em crianças menores de 2 anos, cujas células B ainda não são capazes de responder a este tipo de antígeno (SNAPPER et al., 1995). A conjugação de PS com proteínas

carreadoras visa contornar esse mecanismo, pois é capaz de recrutar células T-específicas para as proteínas e torna os PS imunogênicos em crianças.

A primeira vacina conjugada usada contra pneumococo foi licenciada em 2000, sob o nome comercial de Prevnar (Wyeth) e continha PS de 7 sorotipos (4, 6B, 9V, 14, 18C, 19F e 23F) conjugados à toxina diftérica mutada, CRM₁₉₇. Essa formulação mostrou-se eficaz contra doença invasiva em crianças abaixo dos 2 anos (HANSEN et al., 2006), sendo também capaz de reduzir os níveis de colonização pelos sorotipos vacinais (GHAFAR et al., 2004), o que poderia contribuir com a redução na transmissão da bactéria pelo efeito rebanho. No entanto, essa vacina apresentou cobertura reduzida nos países em desenvolvimento (cerca de 60%), pois não continha em sua formulação dois dos sorotipos mais prevalentes nessas regiões, os sorotipos 1 e 5 (BRANDILEONE et al., 2003). Além disso, após alguns anos da introdução da vacina 7-valente, observou-se uma alteração no espectro de prevalência dos isolados na população, favorecendo o surgimento de sorotipos raros não incluídos na vacina, com consequente redução na eficácia vacinal (FEIKIN et al., 2013; HICKS et al., 2007; HSU et al., 2010; LEHMANN et al., 2010; WEINBERGER; MALLEY; LIPSITCH, 2011).

No ano de 2009, duas novas formulações foram licenciadas e estão disponíveis para uso, sendo uma vacina 10-valente (PCV10 - Synflorix – GSK), que contém 8 polissacarídeos (1, 4, 5, 6B, 7F, 9V, 14 e 23F) conjugados à proteína D de *H. influenzae*, o polissacarídeo 18C conjugado ao toxóide tetânico e o polissacarídeo 19F conjugado ao toxóide diftérico (VESIKARI et al., 2009), e uma formulação 13-valente contendo 13 polissacarídeos (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F e 23F) conjugados ao toxóide diftérico, CRM₁₉₇ (Prevnar13-Wyeth) (FRENCK, 2011); essa vacina entra como substituta para a Prevnar7 e foi licenciada também para uso em adultos acima de 50 anos em alguns países .

A vacina PCV-10 foi introduzida no Programa Nacional de Imunização e está disponível no Sistema Único de Saúde (SUS) para crianças menores de 2 anos, obedecendo os seguintes esquemas de imunização (BRASIL, 2010).

- **3+1:** Três doses para crianças entre 6 semanas e 6 meses, com intervalos de 2 meses, seguida por uma dose de reforço 6 meses após a terceira dose;
- **2+1:** Duas doses para crianças entre 7 e 11 meses, com intervalo de 2 meses, seguida por uma dose de reforço 6 meses após a segunda dose;
- **1:** Uma dose única para crianças entre 12 e 23 meses.

Estudos recentes têm avaliado o impacto desta vacina no Brasil. Em Goiânia, uma pesquisa em crianças entre 7-11 meses ou 15-18 meses, revelou após a vacinação com 2 ou 3 doses de PCV-10, uma redução na colonização de 35,9% e 44%, respectivamente. É importante

ressaltar que entre os sorotipos identificados na nasofaringe, apenas 35,2% estão incluídos na vacina PCV-10, sendo eles o 6B (11,6%), 23F (7,8%), 14 (6,8%) e 19F (6,6%), contra 53% encontrados na PCV-13, onde inclui-se os sorotipos 6A (9,8%) e 19 A (6,3%), foram encontrados também sorotipos não vacinais como 6C (5,9%), 35B (4,3%), 11A (4,3%) e 15 C (3,3%) (ANDRADE et al., 2014). Avaliando o impacto dessa vacinação no número de hospitalização, observou-se que após um ano da introdução da PCV-10 no SUS, houve uma redução média de 26,4% no número de internações causadas por pneumonia, entre crianças de 2 meses e dois anos, nas cidades de Belo Horizonte, Curitiba e Recife, não havendo redução nas cidades de São Paulo e Porto Alegre (AFONSO et al., 2013). No entanto, quando avaliado o impacto nacional após 2 anos de vacinação, na internação de crianças entre 0 e 4 anos, esse valor é reduzido para 12,65% (SCOTTA et al., 2014).

As vacinas conjugadas oferecem proteção restrita aos polissacarídeos presentes na formulação e mesmo com a inclusão de mais sorotipos nas novas formulações, estudos sugerem que uma nova substituição de sorotipos possa ocorrer ao longo dos anos (BEN-SHIMOL et al., 2014; PRYMULA et al., 2011; RICHTER et al., 2014). Além disso, o alto custo das vacinas conjugadas limita sua introdução nos países em desenvolvimento. Assim, apesar do esforço de Agências como “Global Alliance for Vaccines and Immunisation” (GAVI) em introduzir vacinas pneumocócicas em cerca de 46 dos países mais pobres, doenças pneumocócicas continuam liderando as causas de mortes de crianças menores de 5 anos entre as doenças prevenidas por vacinação (Figura 5). Esses dados reforçam a necessidade de se desenvolver vacinas com maior cobertura e custo reduzido.

1.3 Proteínas pneumocócicas como antígenos vacinais

Diversas proteínas pneumocócicas têm sido estudadas como candidatos vacinais contra *S. pneumoniae* e algumas já foram submetidas a ensaios clínicos. Destas, PspA e derivados de Ply apresentam os resultados mais promissores em diversos modelos, quando utilizadas sozinhas ou combinadas, conforme descrito a seguir.

Figura 5. Incidências de morte anuais por doenças vacináveis. (World Health Organization, 2014)

1.3.1 Proteína de superfície de *Pneumococo* A (PspA)

PspA é um fator de virulência exposto na superfície da bactéria, presente em todos os isolados descritos de *S. pneumoniae*, que foi sugerida como antígeno vacinal através de ensaios com anticorpos monoclonais capazes de proteger camundongos de um desafio pneumocócico (MCDANIEL et al., 1986). A proteína madura apresenta quatro domínios principais, mostrados na Figura 6 (JEDRZEJAS; LAMANI; BECKER, 2001). A região N-terminal contém aproximadamente 370 aminoácidos e corresponde à porção funcional de PspA composta pelas regiões A, A', B e pela região rica em prolina. A região A é uma região altamente conservada, seguida pela região variável A'. A região B, embora variável, obedece a padrões de recombinação em blocos ou mosaicos e é também chamada de “Região definidora de clados” (CDR). Esta é seguida por uma região rica em prolinas e pelo domínio de ligação à colina. Este último é responsável pelo ancoramento da PspA a resíduos de fosforilcolina (Chop) presentes no ácido teicóico da parede celular e também nos resíduos de ácido lipoteicóico que estão associados à bicamada lipídica da membrana celular. A extremidade C-terminal inclui uma cauda hidrofóbica curta (BERGMANN; HAMMERSCHMIDT, 2006; BRILES et al., 2000a; GOR et al., 2005).

Figura 6. Esquema linear da PspA. A região N-terminal é composta pelas regiões A (A e A') e região B ou CDR que formam a alfa-hélice em coiled coil, seguida pela região rica em prolinas (PRR). A região C-terminal contém a região de ligação à colina. Adaptado de HOLLINGSHEAD et al., 2000).

Baseado nas variações da CDR, a PspA foi dividida em 3 famílias, subdivididas em 6 clados, de acordo com o grau de identidade (família 1, com clados 1 e 2, família 2, clados 3 a 5, e família 3, clado 6). Entre famílias, a diferença de identidade nesta região é superior a 45%, e entre clados, 20% (HOLLINGSHEAD; BECKER; BRILES, 2000). As PspAs de famílias 1 e 2 (especialmente os clados 1 a 4) são predominantes em todo o mundo (BEALL et al., 2000; MOLLERACH et al., 2004; VELA CORAL et al., 2001); no Brasil, estão presentes em 99% dos isolados clínicos (BRANDILEONE et al., 2004; PIMENTA et al., 2006). Predições de estrutura da PspA sugerem que as regiões A e B formam uma α -hélice predominantemente coiled-coil, seguida pela região C em β -turns (SINGH et al., 2010).

A principal função de PspA envolve a inibição da deposição de complemento na superfície bacteriana, que protege o pneumococo da fagocitose pelo sistema imune (DARRIEUX et al., 2008; REN et al., 2004; TU et al., 1999). Além disso, por ligar-se ao sítio ativo da apolactoferrina, um importante componente da resposta imune inata, a PspA protege o pneumococo da ação bactericida desta proteína (SHAPER et al., 2004). Estudos demonstraram que anticorpos contra PspA são capazes de bloquear suas funções de inibição do sistema complemento, levando a um aumento na deposição de C3 na bactéria e “clearance” pelo sistema imune; soro anti-PspA também é capaz de favorecer a lise da bactéria mediada por lactoferrina (REN et al., 2004; SHAPER et al., 2004), sugerindo a PspA como importante candidato vacinal.

A região N-terminal da PspA contém a maior parte dos epítopos imunogênicos (MCDANIEL et al., 1994) e é a região utilizada na maioria dos estudos vacinais com PspA. Recentemente, foi demonstrado que a “Região rica em prolinas” também está acessível a anticorpos e é capaz de induzir proteção com reatividade cruzada entre clados (DANIELS et al., 2010). A proteção induzida pela imunização com PspA tem sido testada em diferentes

modelos de colonização, pneumonia e doença invasiva (ARULANANDAM et al., 2001; BRILES et al., 2003; DARRIEUX et al., 2007; FERREIRA et al., 2010; MORENO et al., 2011; ROCHE et al., 2003). Kono e colaboradores demonstraram que a PspA é capaz de proteger passivamente filhotes de camundongos através da imunização materna, onde a transferência de anticorpos ocorre pela placenta e/ou leite materno (KONO et al., 2011).

Apesar das variações sorológicas observadas entre os diferentes clados e famílias, a imunização de humanos com uma PspA de clado 2 induziu a produção de anticorpos capazes de reconhecer cepas expressando PspAs de outros clados e famílias. Acredita-se que esse resultado deva-se a um efeito “*booster*” uma vez que virtualmente todos os indivíduos são colonizados por pneumococo ao longo da infância, e desenvolvem anticorpos anti-PspA (NABORS et al., 2000). Em camundongos, foi observado que diferentes moléculas de PspA podem induzir diferentes níveis de reatividade cruzada (DARRIEUX et al., 2008; MORENO et al., 2011). Para uma ampla cobertura vacinal, tem sido proposto que ao menos duas moléculas de PspA (uma de família 1 e uma de família 2) sejam incluídas na formulação vacinal, tornando necessária a escolha de moléculas capazes de induzir ampla reatividade cruzada.

1.3.2 Pneumolisina

Pneumolisina (Ply) foi a primeira proteína a ser descrita como antígeno vacinal contra pneumococo (PATON; LOCK; HANSMAN, 1983), é um membro da família de toxinas ativadas por tiol (BERRY et al., 1995), que apresenta inúmeros efeitos inflamatórios. Ply reconhece e liga-se ao colesterol presente em membranas de células eucarióticas, onde sofre oligomerização, formando poros que levam à lise da célula alvo (GILBERT, 2010).

A Ply é uma proteína de 53 kDa, composta por 4 domínios ricos em folhas β (Figura 7). Primeiramente foi descrita como uma proteína citosólica, sendo liberada para o meio externo por autólise do pneumococo ou em algumas cepas, por mecanismos de exportação ainda não esclarecidos (BALACHANDRAN et al., 2001; BERRY et al., 1989). Outros estudos têm demonstrado que a Ply aparece também associada à parede celular, entretanto, não está acessível a anticorpos (PRICE; CAMILLI, 2009; PRICE; GREENE; CAMILLI, 2012).

Figura 7. Modelo da estrutura da Ply. A proteína é composta por 4 domínios ricos em folhas- β , o domínio 4 está relacionado com sua atividade citolítica e ativação de complemento (ROSSJOHN et al., 1998).

A Ply interfere com funções específicas do sistema imunológico, medeia a expressão de citocinas pró-inflamatórias como IL-1 β , IL-6, TNF- α , e sua instilação no pulmão de ratos foi capaz de reproduzir o processo inflamatório causado pela bactéria (BENTON; VANCOTT; BRILES, 1998; RUBINS et al., 1992). Como mecanismo de evasão, ensaios *in-vitro* demonstraram que a Ply é capaz de inibir o “burst” respiratório de leucócitos polimorfonucleares, reduzindo sua ação bactericida. (JOHNSTON, 1981; PATON; FERRANTE, 1983). Atua também em células do epitélio respiratório, onde inibe os batimentos ciliares reduzindo a eliminação mecânica do pneumococo (FELDMAN et al., 1991). A Ply é capaz de ativar complemento *in-vitro* e está relacionada com a depleção de complemento durante a infecção, reduzindo a capacidade opsonizante do soro (ALCANTARA; PREHEIM; GENTRY-NIELSEN, 2001; PATON; ROWAN-KELLY; FERRANTE, 1984). O mecanismo pelo qual a Ply ativa a cascata do sistema complemento não está muito bem esclarecido; diferentes trabalhos sugerid que a Ply possua estrutura semelhante à proteína C-reativa que ligaria C1q ou ainda que seu domínio 4 possua uma estrutura homóloga à porção Fc de anticorpos e seria responsável pela ativação da via clássica do sistema complemento (PATON; ROWAN-KELLY; FERRANTE, 1984; ROSSJOHN et al., 1998; SHRIVE et al., 1996).

Devido à sua toxicidade na forma nativa, diversas formas detoxificadas (pneumolisóides) foram produzidas por mutagênese sitio dirigida ou detoxificação química. (ALEXANDER et al., 1994; BERRY et al., 1995; DENOEL et al., 2011; PATON et al., 1991; SALHA et al., 2012). O PdT é um pneumolisóide produzido por mutagênese. Possui uma mutação no sítio de ativação de complemento Asp385Asn e duas mutações no domínio 4, responsável pela ligação da pneumolisina em resíduos de colesterol da membrana plasmática, Cys428Gly + Trp433Phe; juntas essas mutações inibem 100% da ativação do complemento e 99,9999% da atividade citolítica (BERRY et al., 1995; MITCHELL et al., 1991).

A imunização com Ply ou pneumolisóides tem se mostrado protetora em camundongos quando desafiados por via intranasal ou intraperitoneal, enquanto a pré-incubação da Ply com anticorpos neutralizantes inibem sua capacidade de induzir inflamação quando instilada no pulmão. (ALEXANDER et al., 1994; KIRKHAM et al., 2006; PATON; LOCK; HANSMAN, 1983; SALHA et al., 2012).

Malley e colaboradores, demonstraram que a Ply promove de forma sinérgica a resposta inata contra o pneumococo através de sua interação com *Toll like receptor-4* (TLR4), promovendo a translocação do NF- κ B em macrófagos derivados de camundongos C57BL/6 o que não ocorre em macrófagos deletados dessa molécula. Além disso, camundongos deficientes para TLR-4 mostraram-se mais susceptíveis a infecção pneumocócica e morte após desafio nasal. Esses resultados sugerem um importante papel da interação da Ply com TLR-4, destacando um possível efeito adjuvante desta molécula. (MALLEY et al., 2003).

Estudos demonstraram também o papel da Ply na ativação do inflamassoma NLRP3, levando à ativação de Caspase 1 seguida pelo processamento e liberação de IL-1 β . Essa ativação mostrou-se essencial na proteção de camundongos durante a infecção pneumocócica. Ainda neste trabalho, McNeela et al. demonstrou o efeito adjuvante da Ply, favorecendo a ativação de células dendríticas com maior expressão de moléculas como CD80 e CD86 e maior produção de anticorpos contra antígenos coadministrados de forma independente de TLR4 (MCNEELA et al., 2010).

Pneumolisóides têm sido testados com sucesso como proteínas carreadoras de diferentes polissacarídeos; - a conjugação de pneumolisóides com PS 19F mostrou-se eficaz em converter a resposta contra o polissacarídeo para uma resposta T dependente e induziu proteção em camundongos (LEE et al., 1994; PATON et al., 1991). Recentemente foi demonstrado que a fusão PsaA-PdT conjugada com Polissacarídeo C é capaz de induzir anticorpos para os três componentes. O conjugado trivalente foi capaz de reduzir a colonização da nasofaringe quando administrado por via intranasal, independentemente da produção de

anticorpos. No entanto, acredita-se que, havendo a invasão de sítios estéreis pelo pneumococo, os anticorpos oferecem uma via adicional de defesa. A administração do conjugado por via subcutânea foi capaz de induzir proteção superior à coadministração dos três componentes (LU et al., 2009). Estes dados reforçam o potencial vacinal de formulações contendo proteínas fusionadas e a possível conjugação com polissacarídeos.

Pneumococos mutantes negativos para PspA e Ply mostraram um aumento significativo na deposição de C3 na sua superfície, demonstrando um efeito sinérgico dessas proteínas na inibição da opsonização (YUSTE et al., 2005). Em outro estudo, a combinação de um pneumolisóide com PspA aumentou a sobrevivência de animais imunizados ativa e passivamente quando desafiados intraperitonealmente (OGUNNIYI et al., 2000). Esses dados sustentam a utilização desses antígenos em uma formulação vacinal. Embora pneumolisóides não induzam anticorpos opsonizantes, sua combinação com PspA seria capaz de induzir anticorpos capazes de neutralizar as propriedades biológicas da toxina, e juntamente com os anticorpos opsonizantes anti-PspA favoreceriam a opsonofagocitose da bactéria.

1.3.3 SP 0148 e SP 2108 – Proteção contra pneumococo mediada por IL-17

Diferentes mecanismos têm sido propostos como eficazes na proteção contra o pneumococo; - utilizando o modelo animal de imunização intranasal seguido por desafio de colonização, Malley e colaboradores demonstraram ser necessária, para a proteção neste modelo, a presença de células CD4⁺ e IL-17 de forma independente de anticorpos (MALLEY et al., 2005; TRZCINSKI et al., 2005). Assim, o mesmo grupo desenvolveu um trabalho avaliando uma biblioteca de expressão de proteínas pneumocócicas da cepa TIGR4 capazes de serem reconhecidas por células Th17, selecionando algumas proteínas potencialmente protetoras. Entre elas a SP 0148 e SP 2108 mostraram resultados promissores (MOFFITT et al., 2011).

A SP 0148 é uma proteína de 276 aminoácidos, denominada proteína ligadora de substrato do sistema de transporte ABC e a proteína SP 2108 é composta por 423 aminoácidos e denominada proteína ligante de maltose. Ambas proteínas são lipídadas, e a resposta imunológica induzida por elas está diretamente relacionada à ativação de TLR-2 (MOFFITT et al., 2014). A imunização de camundongos por via intranasal com SP 0148 e SP 2108, utilizando toxina colérica como adjuvante, foi capaz de induzir a produção de IL-17 em cultura de sangue, após estímulo específico, e conferiu proteção nos camundongos contra colonização por pneumococos de forma dependente de células T CD4⁺ e IL-17. Além disso, essas proteínas

também foram capazes de estimular a produção de IL-17 em culturas de PBMC humanos (MOFFITT et al., 2011).

1.3.4 Candidatos vacinais proteicos em teste clínico

Diversas proteínas foram ou estão sendo avaliadas em diferentes fases de testes clínicos. PspA foi testada em humanos administrada individualmente ou em combinação com PsaA e mostrou-se imunogênica e capaz de induzir anticorpos com ampla reatividade cruzada (BRILES et al., 2000b). Os pneumolisóides PlyD1 e dPly também foram submetidos a ensaios clínicos. PlyD1 foi submetido a ensaio clínico de fase 1 administrado individualmente ou em combinação com as proteínas PhTD/PcpA (ClinicalTrials.gov Identifier: NCT01764126). dPly foi submetida a teste clínico de fase 1 em combinação com PhtD e proteína D de *Hemophilus influenzae* (BERGLUND et al., 2014) e também a ensaio de fase 2 em combinação com a proteína PhTD e a com vacina conjugada PCV10, onde se mostrou segura e imunogênica em crianças entre 12 e 23 meses (PRYMULA et al., 2014). Recentemente, as proteínas SP 0148 e SP 2108 foram avaliadas em ensaio clínico de fase 1, em combinação com uma terceira proteína pneumocócica, onde foram capazes de induzir a produção de anticorpos e IL-17 após 3 doses contendo 100 µg proteínas e Al(OH)₃ como adjuvante (GENOCEA, 2014). O Quadro 1 resume os candidatos vacinais avaliados em teste clínico.

1.4 Adjuvantes

As primeiras vacinas desenvolvidas foram baseadas em patógenos atenuados ou inativados, que contêm inúmeros componentes que atuavam como adjuvantes durante sua administração. As novas gerações de vacinas baseadas em subunidades como polissacarídeos ou ainda proteínas recombinantes são altamente purificadas e muitas vezes falham em ativar uma resposta imunológica efetiva, tornando-se necessário o uso de adjuvantes. Um adjuvante é um componente adicionado à vacina que favorece a resposta imune e pode variar em sua composição química e estrutura, seu uso pode ter como objetivo o aumento da resposta imunológica através do aumento de títulos de anticorpos contra o antígeno utilizado, aumentar a soro conversão em indivíduos com resposta imunológica reduzida, reduzir a quantidade de antígeno necessário ou o número de doses necessárias do protocolo de imunização. Além disso, o uso de adjuvantes pode também direcionar o tipo de resposta imunológica como um aumento

na resposta celular versus resposta humoral. (Revisado em (MOHAN; VERMA; RAO, 2013; SIVAKUMAR et al., 2011)).

Quadro 1. Antígenos proteicos de pneumococos submetidos a ensaios clínicos

Antígenos	Empresa	Fase	Referência
PspA	Sanofi-Pasteur	I	(BRILES et al., 2000b; NABORS et al., 2000) e
PspA + PsaA	Sanofi-Pasteur	I	
PlyD1 monovalente	Sanofi-Pasteur	I	(KAMTCHOUA et al., 2013)
PcpA + PhtD + PlyD1	Sanofi-Pasteur	I	ClinicalTrials.gov Acesso: NCT01764126
dPly + PhtD + Proteína D	GSK	I	(BERGLUND et al., 2014)
dPly e PhtD em combinação com PCV10	GSK	I/II	(PRYMULA et al., 2014)
GEN-004 (SP0148 + SP2108 + SP1912)	GENOCEA	I	(GENOCEA,200
PhtD Monovalente	Sanofi-Pasteur	I/II	(SEIBERLING et al., 2012)
PcpA Monovante	Sanofi-Pasteur	I	(BOLOGA et al., 2012)
PcpA + PhtD	Sanofi-Pasteur	I	
IC-47 (PsaA, PcsB and StkP)	InterCell AG/Novartis/PAT H	I	ClinicalTrials.gov Acesso: NCT00873431
BVH3/11V (Fusão PhpA and PhtB)	IDioMedical (GSK)	II	(HAMEL et al., 2004)

Os adjuvantes baseados em sais de alumínio, que incluem o sulfato potássico de alumínio, sulfato de alumínio e hidróxido de alumínio, vêm sendo utilizados há mais de 80 anos e por muito tempo permaneceram os únicos aprovados para uso em humanos (BAYLOR; EGAN; RICHMAN, 2002; GLENNY, 1930). Recentemente, outras formulações foram liberadas como a série de adjuvantes AS0 licenciados em países da Europa e Estados Unidos e o MF-59 licenciado em países da Europa e recentemente incluído em um estudo clínico aprovado pelo FDA (KHURANA et al., 2014). O Quadro 2 cita os principais adjuvantes e ação.

1.5 BCG como vetor para proteínas heterólogas

O Bacilo de Calmette e Guérin (BCG), uma forma atenuada do *Mycobacterium bovis*, tem sido utilizado há anos como única vacina contra o agente causador da tuberculose – *Mycobacterium tuberculosis* (BEHR, 2002). O BCG é um potente ativador do sistema imunológico, capaz de induzir a produção de citocinas inflamatórias como IFN- γ , IL-2, IL-4, IL-17, quimiocinas e também IL-10, uma citocina regulatória (LALOR et al., 2010; SABLE et al., 2011). Devido às suas propriedades imunomodulatórias, o BCG tem sido estudado também na forma recombinante (rBCG), como vetor vivo expressando antígenos heterólogos de diversos patógenos, como *Leishmania sp.*, *Plasmodium sp.*, *Toxoplasma gondii* (ARAMA et al., 2012; CONNELL et al., 1993; SUPPLY et al., 1999).

Diversos BCG recombinantes têm sido desenvolvidos expressando antígenos de HIV, estes rBCG estão sendo avaliados em diferentes estratégias e modelos animais, especialmente utilizando-se a estratégia de *prime-boost*, onde a segunda dose do antígeno é dada, preferencialmente, utilizando-se um vetor heterólogo (ALDOVINI; YOUNG, 1991; AMI et al., 2005; CAYABYAB et al., 2009; CHAPMAN et al., 2010; SIXSMITH et al., 2014). Resultados promissores, no desenvolvimento de novas vacinas contra MTB, têm sido obtidos pela expressão de citolisinas em cepas de BCG deletadas do locus codificante para a urease C. A expressão da listeriolisina em rBCG incapazes de neutralizar o pH do fagolisossomo, permite que a bactéria escape para o citosol e promova uma melhor apresentação de antígenos e consequentemente, maior proteção no desafio com MTB (CONRADT; HESS; KAUFMANN, 1999; DESEL et al., 2011; REYRAT; BERTUADROHET; GICQUEL, 1995). Uma construção semelhante foi obtida pela inserção do gene para perfringolisina O no locus codificante para a urease C do BCG, esta construção também expressa os antígenos imunodominantes de MTB - 85A, 85B e Rv3407 (SUN et al., 2009). Atualmente essas construções estão sendo avaliadas

em ensaios clínicos sob os nomes VPM 1002 e AERAS-422, respectivamente ((GRODE et al., 2013; KAUFMANN et al., 2014; Clinicaltrial.gov acesso: NCT01340820)).

Quadro 2. Exemplos de adjuvantes utilizados em animais e humanos

Exemplos	Ação	Aprovado para humanos
Hidróxido ou fosfato de alumínio	- Efeito de depósito; - NLP3 - Induz citocinas inflamatórias; - Propicia melhor entrega para APCs; - Forte resposta Th2.	Sim
Hidróxido de Cálcio	Atua como depósito (baixa eficiência)	Somente na Europa
PamCSK	TLR2-	Não
PolyI:C	TLR3	Não
MPL	TLR4	Somente na Europa
Flagelina	TLR5	Não
R848	TLR7/8	Não
CpG DNA	TLR9	Não
Muramyl-dipeptideo	NOD2	Não
Adjuvante de Freund, incompleto ou completo	- Forte resposta Th1 e Th2 - Reduzido efeito de depósito	Não
MF59	- Induz efeito estimulatório local; - Regula expressão de citocinas e quimiocinas Recruta APCs.	Somente na Europa,
Montanide	(APC)	Não
AS0-series	- Ativação local de NF-kB - Indução de Citocinas - Recrutamento de APCs - Resposta Th1	Sim
PLG	APC	Não
ISCOM	- Resposta de células citotóxicas; - Diretamente fagocitado por macrófagos.	Não
Lipossomos Lipossomas baseados na vacina de Hepatite A	Capaz de fundir-se a membrana de macrófagos, favorecendo resposta via MHC I e CD8 citotóxica	Somente na Europa
NB-series	(TLR)	Não
IL-1, IL-2, IL-12	Receptor para citocina	Não
GM-CSF	Receptor para citocina	Não

Adaptado de (SIVAKUMAR et al., 2011; NIAID, 2011)

Trabalhos desenvolvidos em nosso laboratório demonstraram que a imunização com rBCG expressando a subunidade 1 da toxina PT de *Bordetella pertussis* protegeu camundongos contra desafio intracerebral (NASCIMENTO et al., 2000). Além disso, este rBCG mostrou-se eficaz no tratamento de câncer de bexiga em modelo murinho. (ANDRADE et al., 2010). O rBCG expressando o antígeno Sm14 de *Schistosoma mansoni* foi capaz de induzir uma resposta imune específica e protetora também em modelo murino (VARALDO et al., 2004). Recentemente, rBCG expressando intimina ou uma subunidade da “Bundle-forming-pilus” de *E. coli* enteropatogênicas (EPEC) mostrou-se capaz de induzir resposta celular e também bloquear a adesão dessas EPEC às células HEp-2 através de anticorpos de classe IgA ou IgG (VASCONCELLOS et al., 2012).

A construção de um BCG recombinante expressando PspA associada à membrana ou secretada, mostrou-se eficaz na indução de anticorpos específicos contra PspA e foi capaz de proteger camundongos C3H e BALB/c contra desafio letal utilizando-se 100 vezes a DL 50 da cepa WU2 (LANGERMANN et al., 1994). Esses trabalhos demonstram o potencial do BCG como vetor para expressão de diferentes antígenos heterólogos, entre eles proteínas pneumocócicas.

Este trabalho visou desenvolver vacinas baseadas em proteínas pneumocócicas. Na primeira etapa deste projeto, foram selecionadas, através de ensaios de *immunoblotting*, deposição de complemento e opsonofagocitose, duas moléculas de PspA de família 1 capazes de induzir uma ampla reatividade cruzada contra cepas de pneumococo expressando PspAs de clado 1 e 2. Na segunda etapa, a molécula de PspA 2 foi fusionada ao pneumolisóide PdT, e os ensaios funcionais demonstraram que a fusão das proteínas induziu anticorpos com maior afinidade ao pneumococo do que a coadministração das proteínas, promovendo maior opsonização e fagocitose. Estes anticorpos também foram capazes de inibir a atividade citolítica da Ply *in vitro*. Os ensaios de resposta celular demonstraram que a imunização com a proteína híbrida foi mais eficaz na indução de citocinas inflamatórias e protegeu camundongos contra desafio de sepsis.

Com o intuito de aprimorar o mecanismo de apresentação e a resposta imunológica induzida pelos antígenos, construções de BCG expressando PspA2-PdT (rBCG HIb) e outras duas proteínas denominadas SP 0148 (rBCG-0148) e SP 2108 (rBCG-2108) foram desenvolvidas, em colaboração com o Dr. Richard Malley (*Division of Infectious Diseases, Department of Medicine, Children's Hospital, and Harvard Medical School, Boston, MA*). A imunização de camundongo com os BCG recombinantes, isolados ou combinados, mostrou que apenas o rBCG-0148, seguido por uma dose *booster* da mesma proteína recombinante, foi capaz

de induzir níveis significantes de IL-17 na cultura de esplenócitos e reduziu o número de UFC recuperadas após desafio de colonização. Interessantemente, o uso das três vacinas combinadas mostrou-se mais eficiente na proteção contra colonização.

2 OBJETIVOS

2.1 Objetivo geral

Este trabalho teve como objetivo geral desenvolver vacinas pneumocócicas baseadas em proteínas.

2.2 Objetivos específicos

- Selecionar moléculas de PspAs de família 1 (clados 1 e 2), que apresentarem maior reatividade/proteção cruzada dentro desta família;
- Clonar, expressar, purificar, gerar anticorpo e avaliar estes por *immunoblotting*, ligação de anticorpo, inibição da deposição de complemento e ensaio de opsonofagocitose.
- Utilizar os fragmentos selecionados de PspA para obtenção de uma proteína híbrida, contendo os genes de pspA e pdT fusionados;
- Caracterizar a resposta imune e propriedades funcionais da proteína de fusão PspA-PdT utilizando modelo animal;
- Construir cepas de BCG recombinantes expressando as proteínas pneumocócicas PspA-PdT, SP 0148 e SP 2108;
- Avaliar a resposta imune e proteção contra desafio em camundongos imunizados com as cepas de rBCG produzidas.

3 MATERIAL E MÉTODOS

3.1 Cepas de *Streptococcus pneumoniae* e condições de crescimento

As cepas de *Streptococcus pneumoniae* utilizadas neste estudo estão descritas no Quadro 3. As bactérias foram mantidas a -80 °C e antes de cada experimento plaqueadas em meio Agar sangue. Após incubação por aproximadamente 16 h a 37 °C sob condição anaeróbica, as bactérias foram transferidas para meio líquido Todd-Hewitt broth (Becton, Dickinson Bioscience, Franklin Lakes, NJ, EUA – BD) suplementado com 0,5% de extrato de levedura (BD) e incubadas a 37 °C até atingirem densidade óptica a 600 nm (D.O.₆₀₀) entre 0,4 e 0,6.

3.2 Animais

Camundongos BALB/c e C57BL/6 fêmeas de 5 a 7 semanas de idade foram obtidos do biotério de criação da Faculdade de Medicina (FMUSP) e mantidos no biotério de experimentação animal do Laboratório de Biotecnologia Molecular IV segundo as normas da Comissão de Ética no Uso de Animais do Instituto Butantan (CEUA/IB) sob o protocolo 602/09.

3.3 Clonagem, expressão e purificação das proteínas recombinantes

3.3.1 Obtenção dos fragmentos gênicos

Os fragmentos gênicos das diferentes pspAs foram amplificados pela PCR a partir de um painel de 10 cepas de *S. pneumoniae* brasileiros (BRANDILEONE et al., 2004), utilizando-se oligonucleotídeos específicos (Quadro 3). A PCR foi realizada seguindo especificações da enzima DNA Taq polimerase (Thermo Fisher, Waltham, MA, EUA). O fragmento gênico da pdT (pneumolisina detoxificada) foi obtido por PCR utilizando os oligonucleotídeos Forward 5' – GCAAATAAAGCAGTAAATGACTT – 3' e Reverso 5' – ATTTTCTACCTTATCCTC – 3' a partir do plasmídeo pQE-30-pdT, construído pelo Dr. James C. Paton (Research Centre for Infectious Diseases, School of Molecular and Biomedical Science, University of Adelaide, Australia) e cedido pelo Dr. Richard Malley (Division of Infectious Diseases, Department of Medicine, Children's Hospital, and Harvard Medical School, Boston).

3.3.2 Preparação de *E. coli* quimiocompetentes

A preparação da competência, ligação e transformação de *E. coli* DH5- α e BL21 DE3 (Invitrogen, Burlington, CA) e M15 (Qiagen) foram realizadas conforme descrito por Sambrook (1989). A seleção de transformantes das *E. coli* DH5- α e BL21-DE3 foi realizada em meio Luria-Bertani (LB) contendo 100 $\mu\text{g/mL}$ de ampicilina (Sigma) - (LB-amp.) e para M15 utilizou-se LB contendo 20 $\mu\text{g/mL}$ de canamicina (Sigma) e 100 $\mu\text{g/mL}$ de ampicilina (LB-cam-amp), após incubação a 37 °C por aproximadamente 16 h.

3.3.3 Clonagem dos fragmentos gênicos de *pspA*

Os fragmentos gênicos de *pspA* amplificados por PCR foram inseridos no vetor pGEMT-easy (Promega, Fitchburg, WI, EUA). Utilizando-se *E. coli* DH5- α quimiocompetentes transformadas com os respectivos plasmídeos e selecionadas em meio LB-amp; foram realizadas minipreparações das culturas e os plasmídeos obtidos digeridos com as endonucleases *XhoI* e *EcoRI* (Invitrogen). As amostras foram submetidas à eletroforese em gel de agarose 1%, os fragmentos com tamanho esperado purificados e inseridos no vetor de expressão em *E. coli* pAE 6xHis cedido pelo Dr Celso Ramos, previamente digerido com as mesmas endonucleases (RAMOS et al., 2004). Este vetor contém uma sequência para adição de 6 histidinas à extremidade N-terminal da proteína recombinante, o que permite sua posterior purificação por cromatografia de afinidade. Os plasmídeos dos clones crescidos em ampicilina foram extraídos por minipreparações e confirmados por sequenciamento.

3.3.4 Clonagem da fusão *pspA*-*pdT*

O gene mutante foi inserido no vetor de clonagem pGEM-T easy (Promega) - (originando pGEM-T easy – *pdT*) e o fragmento gênico codificante para PspA 94/01 (PspA2), inserido após clivagem da primeira construção com as enzimas *SacI* e *XhoI* (Invitrogen) - (formando pGEMT-easy-*pspA*-*pdT*). A fusão gênica *pspA2-pdT* foi clivada utilizando-se as enzimas de restrição *Sac I* e *Sma I* e inserida no vetor pQE-30 (Qiagen) previamente linearizado com as mesmas enzimas. Este vetor também contém uma sequência para adição de 6 histidinas à extremidade N-terminal da proteína recombinante. A integridade dos fragmentos foi confirmada por sequenciamento.

Quadro 3. Cepas de *S. pneumoniae* utilizadas durante todo estudo.

Cepas	Clado PspA	Serotipo	Origem	Ensaio	Oligonucleotídeos
M12	1	6B	UFG	Clon; IB	a; c
P13	1	9V	UFG	Clon; IB	a; c
P69	1	10A	UFG	Lig.; Comp; OPA	--
P125	1	15B	UFG	Comp	--
P231	1	6A	UFG	IB	--
245/00	1	14	IAL	Lig; Clon; IB; Comp; OPA	a; d
P630	1	14	UFG	Comp	--
P1031	1	23F	UFG	Clon; IB	a; c
491/00	1	6B	IAL	Desafio	--
P1079	1	1	UFG	Clon; IB; OPA	a;c
3JY44182-95	1	3	UAB	OPA	--
M8	2	6B	UFG	IB	--
P94	2	19F	UFG	OPA	--
94/01	2	18A	IAL	Lig; Clon; IB; Comp	a; d
P278	2	18C	UFG	Clon; IB; OPA	b; d
325/95	2	6A	IAL	Clon; IB	b; d
P339	2	6A	UFG	Clon; IB	b; d
373/00	2	6B	IAL	Clon; IB; OPA	b; d
P854	2	19F	UFG	Comp; OPA	--
A66.1	2	3	UAB	Lig; IB; Comp	--
D39	2	2	UAB	IB; Comp	--
TIGR4	3	4	UAB	Clon.	--
WU2	2	3	UAB	Desafio intranasal	--
603	-	6B	BCH	Desafio colonização	--

IAL: Instituto Adolfo Lutz, São Paulo, Brasil.

UFG: Universidade Federal de Goiás, Goiânia, Brasil.

UAB: University of Alabama at Birmingham, EUA.

BCH: Boston Children's Hospital, EUA

Clon: Clonagen do fragment gênico da PspA.

Lig: Ligação de anticorpo

IB: *Immunoblotting*

Comp: Ensaio de deposição de complemento

OPA: Ensaio de Opsonofagocitose

a: PspA – Direto 5' - GAAGCGCCCGTAGCTSGTC - 3'
 b: PspA – Direto 5' - ACCATGGTAAGAGCAGAAGAAGCC - 3'
 c: PspA – Reverso 5' - TTATTCTGGTTTAGGAGCTGGAGCTGG - 3'
 d: PspA – Reverso 5' - CCACATACCGTTTTCTTGTTCAGCC - 3' (HOLLINGSHEAD; BECKER; BRILES, 2000)

3.3.5 Expressão em *E. coli*

E. coli BL21 DE3 (Invitrogen) ou M15 (Qiagen) quimiocompetentes foram transformadas com as construções em vetor pAE e pQE respectivamente, plaqueadas em meio LB-Agar adicionado de antibióticos e incubadas a 37 °C por aproximadamente 16 h para seleção dos clones transformados. Para a expressão foi preparado um pré-inóculo, inoculando-se 1 colônia de *E. coli* em 20 mL de meio 2YT-amp (BL21-DE3) ou 2YT-can-amp (M15) que foi incubado sob agitação a 37 °C, 200 rpm, por aproximadamente 16 h. A expressão foi realizada utilizando 400 mL de meio 2YT adicionado de antibióticos ao qual foi adicionado o pré-inóculo na diluição de 1:20. A indução da expressão foi realizada pela adição de 1 mM de IPTG (Sigma) no cultivo entre D.O.₆₀₀ 0,6 - 0,8 e mantida por 3 h.

3.3.6 Purificação das proteínas recombinantes por cromatografia líquida de afinidade ao Ni²⁺

Após o período de indução, as culturas foram centrifugadas a 3.200 x g durante 10 min a temperatura de 4 °C, os pellets ressuspensos em tampão (50mM Tris, 150mM NaCl e 5 mM Imidazol - Sigma) na proporção 1:10 e lisados no aparelho French Press. Após centrifugação a 8.000 x g, as proteínas recombinantes contidas no sobrenadante foram purificadas por cromatografia de afinidade ao Ni²⁺, com auxílio do aparelho Akta Prime (GE HealthCare, Waukesha, WI, EUA). Após a eluição com gradiente de imidazol as frações foram analisadas por SDS-PAGE, as amostras positivas reunidas e dialisadas contra tampão 10mM Tris-HCl (pH 8), 20 mM NaCl. As proteínas recombinantes foram quantificadas pelo método de Bradford (Bio-Rad, Hercules, CA, UK) e utilizadas na imunização de camundongos.

3.3.7 Análise da expressão e purificação da rPspA-PdT por *immunoblotting*

A proteína híbrida rPspA-PdT purificada, juntamente com a rPspA e rPdT foram separadas por SDS-PAGE e transferidas para membranas de PVDF (GE) (120 mA, 90 min). Após bloqueio realizado com 10% de leite desnatado, as membranas foram incubadas por 2 h com soros anti-PspA2 ou anti- Ply na diluição 1:4000 e 1:2000 respectivamente. Após lavagem com tampão TBS-T (100 mM Tris, 150 mM NaCl e ,05% Tween® 20-Sigma), as membranas

foram incubadas com anticorpo anti-IgG de camundongo conjugado a peroxidase de raiz forte (*horseradish peroxidase* - HRP) (Sigma) na diluição de 1:4.000 durante 1 h. Após nova lavagem, a detecção foi realizada utilizando-se o Kit ECL (GE).

3.3.8 Remoção do LPS por lavagem com Triton X-114

A fim de remover o excesso de LPS presente nas proteínas recombinantes purificadas de *E. coli* as amostras foram submetidas a lavagens consecutivas com Triton X-114 (Sigma). O detergente foi adicionado às amostras de proteínas na concentração final de 1%. As amostras foram misturadas e incubadas por 30 minutos a 4° C por 30. Em seguida, as amostras foram incubadas por 10 minutos a 37 °C e centrifugadas por 10 minutos a 16,000 g a 25° C. A fase aquosa (superior) foi retirada e ressubmetida a mais 2 etapas de lavagem com Triton X-114 (LIU et al., 1997).

3.4 Imunização de camundongos com as proteínas recombinantes

3.4.1 Imunização com PspAs de família 1

Camundongos BALB/c fêmeas entre 5 e 7 semanas foram imunizados por via intraperitoneal com 5 µg de rPspA, utilizando-se 50 µg de hidróxido de alumínio (Al(OH)₃) como adjuvante no volume total de 500 µL de solução salina 0,9%, totalizando 3 doses em intervalos de 7 dias. O adjuvante hidróxido de alumínio diluído em solução salina foi utilizado como controle. Os animais foram sangrados pela via retro-orbital 7 dias após cada imunização. O sangue obtido foi centrifugado a 230 x g, 10 min a 4 °C e o soro armazenado a -20 °C.

3.4.2 Imunização com a proteína híbrida PspA-PdT

Camundongos BALB/c fêmeas entre 5 e 7 semanas foram imunizados por via subcutânea com as proteínas recombinantes descritas no Quadro 4, utilizando-se 50 µg de hidróxido de alumínio (Al(OH)₃) como adjuvante no volume total de 200 µL de solução salina 0,9%, totalizando 3 doses em intervalos de 15 dias. O adjuvante hidróxido de alumínio em solução salina foi utilizado como controle. Os animais foram sangrados pela via retro-orbital 15 dias após cada imunização. O sangue obtido processado como descrito anteriormente.

Quadro 4. Grupos, doses e imunógenos utilizados na imunização de camundongos

Grupo	Dose e imunógeno
1 – Salina	-
2 – rPspA2	8,8 µg rPspA 94/01
3 – rPdT	11,2 µg rPdT
4 – rPspA2 + rPdT (Coad)	8,8 µg rPspA 94/01 + 11,2 µg rPdT
5 – rPspA2-PdT (Híbrido)	20 µg rPspA 94/01-PdT

3.5 Análise da resposta humoral induzida

3.5.1 Avaliação da indução de anticorpos IgG por ELISA

As proteínas recombinantes PspA ou PdT foram imobilizadas separadamente em placas de 96 poços (Nunc International, Rochester NY, EUA) na concentração de 1 µg/mL em tampão Carbonato-Bicarbonato, pH 9,6 (100 µL/poço) e incubadas a 4 °C por aproximadamente 16 h. Foi utilizada IgG de camundongo para obtenção da curva padrão (FERREIRA et al., 2008). As placas foram lavadas 3 x, bloqueadas com 200 µL/poço de 10% de leite desnatado em tampão fosfato salino (PBS) e, após nova lavagem, foram adicionados os soros dos animais imunizados individualmente na diluição inicial de 1:100 (100 µL/poço), seguida por uma diluição seriada e incubação de 2 h. As placas foram lavadas novamente 3 x e incubadas com anticorpos de cabra anti-IgG de camundongo na diluição de 1:10.000 (100 µL/poço). Após nova lavagem, as placas foram incubadas com anticorpos de coelho anti-IgG de cabra conjugado com HRP na diluição de 1:20.000 (100 µL/poço). Todas as lavagens foram realizadas utilizando-se PBS (pH 7,2) + Tween 20 (0,05%) e os anticorpos diluídos em PBS contendo 1% de BSA. A curva padrão e anticorpos utilizados são da empresa Southern Biotech, Birmingham, AL, EUA. A revelação foi realizada utilizando o substrato 0,4 mg/mL de O-fenilenediamina (OPD, SIGMA) diluído em tampão citrato de sódio (100 µL/poço). A reação foi bloqueada com H₂SO₄ 4 M (50 µL/poço) e a absorbância das amostras determinada através de leitura no aparelho Multiskan EX a 492 nm. As concentrações dos anticorpos foram calculadas pela correlação com a curva padrão gerada a partir da diluição de IgG total. Na análise estatística, foi utilizado o teste ANOVA seguido pelo teste de Tukey.

3.5.2 Avaliação da reatividade cruzada por *immunoblotting*

Isolados de *S. pneumoniae* expressando PspAs de família 1 (clado 1 e 2) foram plaqueados como descrito previamente. Em seguida, os cultivos foram inoculados em 50 mL de meio líquido THY e incubados novamente a 37 °C até atingirem D.O.₆₀₀ entre 0,6 - 0,8. As bactérias foram centrifugadas a 3.200 x g durante 10 min. Os pellets foram lavados três vezes com PBS e centrifugados. Em seguida, os pellets foram ressuspensos em 1 mL de PBS contendo 2% de cloreto de colina (SIGMA) e incubados durante 10 min em temperatura ambiente. Após nova centrifugação, o sobrenadante contendo PspA (Extrato de colina) foi coletado, quantificado pelo método de Bradford e armazenado a -20 °C (BRILES et al., 1996).

Amostras contendo 2 µg de extrato de colina obtidos após a lavagem das bactérias com PBS-cloreto de colina foram separadas por SDS-PAGE e transferidas para membranas de nitrocelulose (120 mA, 90 min). Após bloqueio realizado com 10% de leite desnatado, as membranas foram incubadas com pool dos respectivos soros (5 anti-PspAs clado 1 e 5 anti-PspAs clado 2) na diluição 1:1.000 durante 2 h, lavadas com tampão TBS-T (100 mM Tris, 150 mM NaCl e 0,05% Tween® 20) e incubadas com anticorpo secundário de cabra anti-IgG de camundongo - HRP (Sigma) na diluição de 1:1000 durante 1 h. Após nova lavagem, a detecção foi realizada utilizando-se o Kit ECL (GE Healthcare).

3.5.3 Avaliação da ligação de anticorpos e deposição de complemento na superfície do pneumococo

Culturas de pneumococo preparadas como descrito anteriormente, foram centrifugadas (3.200 x g, 5 min), lavadas no mesmo volume de PBS e 100 µL foram incubados na presença de 5% de soro dos animais imunizados (pool nos experimentos de reatividade cruzada e individualmente nos experimentos com a proteína híbrida), previamente incubados a 56 °C durante 30 min para inativação das proteínas do sistema complemento, a 37 °C durante 30 min. Após lavagem com PBS, as amostras foram incubadas com anticorpo anti-IgG conjugado com isotiocianato de fluoresceína (FITC-*conjugated goat anti-mouse IgG*, MP Biomedicals), na diluição de 1:1.000. As amostras foram lavadas 2 X com PBS, fixadas em PBS contendo 1% de formaldeído (SIGMA) e armazenadas a 4 °C, no escuro, até a leitura das células em citômetro de fluxo FACS CANTO (BD). Para avaliar a capacidade desses soros de favorecer a deposição de complemento na superfície do pneumococo, após a incubação com os antissoros as amostras foram centrifugadas e ressuspensas em tampão de opsonização com 5% de soro normal de camundongo (NMS). As amostras foram novamente incubadas a 37 °C por 30 min, lavadas e

incubadas na presença de anticorpos anti-C3 conjugado com isotiocianato de fluoresceína (FITC-*conjugated goat antiserum o mouse complement C3*, MP Biomedicals), na diluição de 1:500, no gelo durante 30 min (REN et al., 2003). A análise dos dados foi realizada com o auxílio do programa Flow Jo e a análise estatística utilizando ANOVA e teste de Turkey.

3.5.4 Ensaio de opsonofagocitose usando células peritoneais murinas

Para obtenção das células peritoneais murinas (macrófagos e neutrófilos) camundongos BALB/c foram estimulados por via intraperitoneal com 10 µg de Concanavalina A (ConA, SIGMA). Após 48 h, os animais foram sacrificados e submetidos a um lavado intraperitoneal utilizando-se 5 mL de PBS gelado (RODRIGUES et al. 1992). As células foram mantidas em gelo até o uso.

S. pneumoniae de família 1 foram cultivados conforme descrito anteriormente. Após atingirem a D.O.₆₀₀ entre 0,4 e 0,5 as bactérias foram centrifugadas a 3.200 x g por 5 min lavadas com PBS e ressuspensas em tampão de opsonização (Solução-tampão salina de Hank adicionado de cálcio e magnésio (HBSS - Gibco, CA, EUA) e 0,1% de gelatina (SIGMA), (STEINER, 1999). Alíquotas contendo aproximadamente $2,5 \times 10^6$ unidades formadoras de colônias (UFC) foram incubadas com pool dos soros dos animais imunizados ou soro de animal injetado (previamente inativados por calor), na diluição de 1:8 e 1:16 a 37 °C por 30 min. Após este período, as amostras foram centrifugadas a 3.200 x g por 5min, lavadas com PBS e incubadas com 10% de soro normal de camundongo em tampão de opsonização e novamente incubada a 37 °C por 30 min. Em seguida as amostras foram lavadas com PBS e incubadas com 4×10^5 células peritoneais murinas diluídas em tampão de opsonização e incubadas a 37 °C a 200 rpm por 45 min. A reação foi bloqueada por incubação em gelo por 5 min. As amostras foram submetidas a diluições seriadas, plaqueadas em Agar sangue e o número de UFC recuperadas contado após 18 h. Foram preparadas também lâminas por *cytospin* e coradas usando Instant-Prov (Newprov).

3.5.5 Ensaio de inibição de hemólise

A Ply recombinante foi expressa a partir do clone de *E. coli* M15 - RM 86 gentilmente cedido pelo Dr. Richard Malley. A atividade da Ply foi testada através do ensaio de hemólise (PATON; LOCK; HANSMAN, 1983). A solução de 2% de hemácias de carneiro em PBS foi preparada utilizando-se 200 µL de sangue de carneiro, lavado 3 x com 1 mL de PBS e

ressuspendido em 10 mL de PBS. O ensaio de hemólise foi preparado em placas de 96 poços, onde foi adicionado 1 µL da Ply purificada em 49 µL de PBS, seguida por diluição seriada e em seguida foi acrescentado 50 µL da solução de hemácias 2%. Após incubação por 30 min a 37 °C, as amostras foram centrifugadas a 450 x g por 10 min e o sobrenadante transferido para outra placa. A absorbância do sobrenadante foi determinada por leitura em Multiskan EX a 540nm. Foi utilizada água e uma proteína inespecífica, preparada nas mesmas condições da Ply como controle da purificação e diálise. A atividade da Ply foi expressa em unidades hemolíticas (UH) que é determinada pela quantidade de Ply necessária para a hemólise de 50% das hemácias.

Para o ensaio de inibição de hemólise 8 UH de Ply foram incubadas com soros dos animais imunizados com PspA2, PdT, PspA2+PdT e PspA2-PdT na diluição de 1:40 (volume final de 50 µL) por 30 min a 37 °C. Em seguida foi adicionado 50 µL da solução de hemácias 2% e o experimento seguido conforme descrito anteriormente. A porcentagem de hemólise foi calculada com base na hemólise obtida pela Ply incubada com soro dos animais que receberam somente Al(OH)₃ que foi considerada como 100%.

3.6 Avaliação da resposta celular induzida pela imunização com PspA2-PdT

3.6.1 Cultura celular e avaliação da produção de citocinas

Para os ensaios de resposta celular, os animais foram imunizados com PspA2, PdT, proteínas coadministradas ou proteína híbrida PspA2-PdT adsorvidas em Al(OH)₃, MPLA (Sigma) ou na ausência de adjuvantes. Após 15 dias da terceira imunização, os animais foram sangrados e eutanasiados para coleta do baço. Para comparação da resposta celular antes e depois do desafio, animais imunizados utilizando-se Al(OH)₃ como adjuvante foram desafiados por via intraperitoneal com 5 x 10⁶ UFC da cepa 245/00 e eutanasiados após 24 h. Os baços foram processados individualmente em meio RPMI (Invitrogen). A porção contendo as células foi centrifugada a 450 x g por 10 min e o sobrenadante descartado. As hemácias presentes no pellet celular foram lisadas por incubação com 1 mL de água destilada estéril durante 10 s, seguida por adição de 10 mL de RPMI (Invitrogen) e nova centrifugação. O pellet celular foi então ressuspendido em 1 mL de RPMI (Invitrogen), e a viabilidade celular analisada por contagem em câmara de Neubauer utilizando-se azul de tripan. A cultura celular foi realizada em placas de 96 wells (Corning-Costar) de fundo U utilizando 1 x 10⁶ células/poço em 100 µL de meio RPMI contendo 10% de soro fetal bovino (Sigma) - RPMI complemento. Para o

estímulo das células, utilizou-se 5 µg/mL de PspA2 ou PdT (estímulos específicos), ou Concanavalina A (ConA - estímulo inespecífico). A cultura de células incubadas somente com meio RPMI completo foi utilizada como controle. A produção de citocinas foi avaliada utilizando-se o Kit CBA (Cytometric Bead Array) perfil Th1, Th2, Th17, seguindo as especificações do fabricante (BD-Bioscience). Os dados foram adquiridos em FACS Canto II (BD) e analisados no Software FCAP 3.0.

3.7 Ensaio de proteção pela imunização com proteínas recombinantes

3.7.1 Desafio letal intravenoso

Após 15 dias da terceira imunização, os animais imunizados com PspA2, PdT, proteínas coadministradas ou proteína híbrida PspA2-PdT, receberam uma dose letal de pneumococo contendo 5×10^6 UFC da cepa 491/00 (PspA clado 1) por via intravenosa. Os animais foram monitorados por 15 dias e a sobrevivência entre os grupos analisada utilizando Mann–Whitney U test.

3.8 Expressão de proteínas pneumocócicas em BCG

3.8.1 Preparação do BCG eletrocompetente

Para preparação da BCG eletrocompetente, uma única colônia foi inoculada em meio Middlebrook-7H9 (Difco), contendo glicerol 0,5% (Sigma) e Tween 80 0,05% (Inlab) e suplementado com 10% de OADC (ácido oleico, albumina, dextrose e catalase) - (MB7H9-OADC) e mantido a 37 °C e 5% de CO₂ até D.O.₆₀₀ 0,6 – 0,8. O cultivo foi centrifugado a 3200 g a 4 °C por 10 min. O sobrenadante foi descartado e o sedimento celular foi lavado três vezes com 50 mL, 10 mL e 5 mL de glicerol 10% gelado estéril, removendo o sobrenadante por centrifugação nas mesmas condições anteriores. Ao final, o sedimento celular foi ressuspensionado em 1 mL de glicerol 10% e aliquoteado em tubos de 50 µL. As alíquotas foram estocadas a -80 °C até seu uso.

3.8.2 Construções de vetor de expressão em micobactérias expressando proteínas pneumocócicas

O fragmento gênico codificante para proteína híbrida PspA2-PdT, previamente amplificado, e os fragmentos de SP 0148 e SP 2108, clonados a partir do DNA genômico da cepa pneumocócica TIGRE4 (MOFFITT et. al., 2011), foram inseridos no vetor episomal de expressão em micobactérias pMIP12, cedido pela Dra Brigitte Gicquel (Instituto Pasteur – Paris, França). As construções pMIP-*pspA2-PdT*, pMIP-SP 0148 e pMIP-SP 2108 foram confirmadas por sequenciamento e utilizadas na transformação das cepas de BCG da linhagem Pasteur.

3.8.3 Cultura do BCG

O BCG eletrocompetente foi transformado por eletroporação após a adição de 100-300 ng de cada construção previamente descrita, nas seguintes condições: 2,5 kV, 25 μ F e 1000 Ω em eletroporador de pulsação Gene Pulser II (Bio-Rad). A eficiência da eletroporação foi monitorada observando a constante de tempo, usualmente entre 17 – 19 ms para BCG. Após o pulso, a cubeta foi incubada em gelo por 10 min e as células, ressuspensas em 1 mL de MB7H9-OADC e pré-incubadas a 37 °C por 16 h. As amostras foram plaqueadas em meio MB7H10 suplementado com OADC e 20 μ g/mL de canamicina e incubados a 37 °C em estufa com atmosfera a 5% de CO₂ por aproximadamente 30 dias. Após o período de incubação, as colônias crescidas na placa foram transferidas para meio líquido MB7H9-OADC contendo 20 μ g/mL de canamicina e mantidas a 37 °C em estufa com atmosfera a 5% de CO₂ até atingirem D.O.600 entre 0,6-0,8. As culturas foram centrifugadas, lavadas com PBS 1 X, aliquotadas em tubos de 100 μ L e estocadas a -80 °C.

3.8.4 Avaliação da expressão das proteínas pneumocócicas em BCG recombinante

Para análise da expressão das proteínas heterólogas, uma alíquota de cada amostra foi ressuspensa em 500 μ L de PBS 1 X contendo inibidor de proteases PIC (Sigma) e lisada por sonicação sob gelo numa amplitude de 60 Hz (Ultrasonic Processor GE 100) por 5 min utilizando pulsos de 1 s. Após centrifugação a 10,000 g por 30 min a 4 °C as amostras foram separadas em fração solúvel (sobrenadante) e fração insolúvel (pellet) e quantificadas pelo método de Bradford (Bio-Rad). Amostras contendo 30 mg da fração solúvel ou 10 mg da fração

insolúvel foram submetidas a SDS-PAGE, transferidas para membrana de PVDF (GE) e o *immunoblotting* realizado como descrito anteriormente, utilizando-se anticorpos específicos.

3.9 Avaliação da resposta imunológica induzida pela imunização com os rBCG

3.9.1 Imunização de camundongos

Para avaliação da resposta imunológica induzida pelos BCG recombinantes, camundongos C57BL/6 foram imunizados com uma dose contendo 1×10^6 UFC do BCG controle ou de cada BCG recombinante – rBCG-0148, rBCG-2108 ou rBCG Hib – para o BCG Mix foram utilizadas 3×10^5 de cada BCG recombinante. Para os grupos imunizados com a vacina pneumocócica celular não encapsulada foram utilizados 100 µg por dose. O *booster* com proteínas recombinantes foi realizado 36 dias após a dose de BCG, nas seguintes concentrações: - rSP-0148 – 1 µg; - rSP 2108 – 3 µg; PspA-PdT – 20 µg/ animal utilizando 100 µg de Al(OH)₃ como adjuvante. Os animais foram sangrados 30 dias após a dose de BCG ou 15 dias após a dose *booster* de proteínas e o sangue obtido utilizado para a cultura de sangue total e obtenção de soro para posterior análise de anticorpos.

3.9.2 Avaliação de citocinas em cultura de sangue total

A cultura de sangue foi realizada utilizando 10 % (25 µL) de cada amostra de sangue total heparinizada adicionadas a 225 µL de meio DMEM/F12 (Gibco) suplementado com 10% de soro fetal bovino (Sigma), 2 mM de L-glutamina (Sigma), 50 mM de β-mercaptoetanol (Sigma) e 10 µg/mL de ciprofloxacina (Sigma) (MOFFITT et al., 2011). A cultura foi incubada em estufa de CO₂ por 6 dias e após este período o sobrenadante coletado para análise de citocinas por ELISA como descrito pelo fabricante (RD ou Peprotec).

3.9.3 Avaliação de citocinas em cultura de esplenócitos

Após 21 dias da dose *booster* de proteínas recombinantes os animais foram eutanasiados e o baço coletado para cultura de esplenócitos como descrito anteriormente. Foram utilizados 5 µg/mL de rSP 0148, rSP 2108, rPspA2 ou 10 µg/mL de PdT como estímulos. A cultura foi mantida em estufa de CO₂ e o sobrenadante coletado após 48 h. A análise de

citocinas foi realizada utilizando-se Kit CBA Th1, Th2, Th17 (BD) como descrito anteriormente.

3.10 Ensaio de proteção pela imunização com rBCG

3.10.1 Desafio letal por pneumonia

O desafio letal por pneumonia foi realizado após 21 dias da dose *booster* de proteínas recombinantes. Os animais anestesiados receberam por via intranasal 1×10^6 ou 1×10^7 UFC de cepa de pneumococo WU2 diluídas em 50 mL de PBS 1X. Os animais foram monitorados por 15 dias e os animais que sobreviveram ao desafio sangrados para a avaliação da presença de bactérias no sangue por plaqueamento em ágar sangue.

3.10.2 Desafio de colonização

Para colonização da nasofaringe, os animais não anestesiados receberam por via intranasal 1×10^6 UFC da cepa pneumocócica 603 diluída em 20 μ L de PBS 1X. Após uma semana, os animais foram eutanasiados e o lavado nasal realizado com o auxílio de uma cânula por meio da inoculação de PBS através da traqueia. As 6 primeiras gotas eliminadas pelas narinas foram coletadas e plaqueadas em ágar sangue, nas diluições de 1:5, 1:25 e 1:125 ou sem diluição. As UFC recuperadas foram contadas após 16 h de incubação a 37 °C em atmosfera anaeróbica (MALLEY et al., 2001).

4 RESULTADOS

4.1 Análise da reatividade cruzada entre PspAs de família 1

4.1.1 Expressão e purificação de rPspAs:

Para obtenção dos fragmentos de PspA de família, que seriam utilizados nos posteriores testes de reatividade cruzada, fragmentos gênicos de PspA de 10 cepas de pneumococos brasileiros foram clonados e as proteínas expressas em *E.coli*, em fusão com uma cauda de 6 Histidinas e purificadas por afinidade a Ni^{2+} . A análise por SDS-PAGE das rPspAs purificadas é mostrada na Figura 8. Pode-se verificar que o peso molecular das proteínas recombinantes produzidas variou entre aproximadamente 45 e 70 kDa, esse resultado deve-se primeiramente pela heterogeneidade dessas proteínas que podem variar entre 42 e 90 kDa devido a presença ou não da região Non-Pro na porção C-terminal da proteína. Além disso, a região rica em prolinas, também presente na porção C-terminal favorece o alinhamento do oligonucleotídeo reverso em diferentes posições, levando então a obtenção de proteínas com tamanhos diferentes.

Figura 8. SDS-PAGE das PspAs recombinantes purificadas. A região N-terminal de 10 PspAs de família 1 foram expressas em *E. coli* em fusão com uma cauda de histidina e purificadas por cromatografia de afinidade a Ni^{2+} . Padrão de massa molecular (kDa) é indicado à esquerda.

4.1.2 Avaliação da reatividade cruzada induzida por soros anti-PspAs por *immunoblotting*

A primeira etapa da avaliação da reatividade cruzada entre PspAs de família foi realizada através de análises por *immunoblotting*. O soro de camundongos BALB/c imunizados com 3 doses da rPspAs foi testado quanto à capacidade de reconhecer PspAs nativas, extraídas através de lavagens com cloreto de colina (Extrato de Colina) de pneumococos de família 1. Foram utilizadas 6 cepas de pneumococo expressando PspAs de clado 1, e 7 cepas expressando

PspAs de clado 2 e os resultados obtidos são mostrados na Figura 9. As análises revelaram uma variação significativa no nível de reatividade cruzada entre soros obtidos de rPspAs de clado 1 e 2. Entre os soros avaliados, 4 apresentaram altos níveis de reatividade cruzada com PspA de ambos os clados, sendo dois de clado 1 – anti-PspA M12 e anti-PspA 245/00 – e dois de clado 2 – anti-PspA94/01 e anti-PspA P339.

Figura 9 Análise da reatividade cruzada de soros anti-PspAs por *immunoblotting*. Soros policlonais de camundongos imunizados com a região N-terminal de 10 PspAs recombinantes de família 1 (5 de cada clado) foram testados quanto a capacidade de reconhecer diversos extratos de colina obtidos de pneumococos expressando PspAs de clado 1 e 2 (diluição 1:1000). O padrão de massa molecular (κDa) está indicado à esquerda e os controles à direita (sublinhados).

4.1.3 Deposição da proteína C3 do sistema complemento na presença de anticorpos anti-PspA selecionados por *immunoblotting*

Os soros anti-PspAs selecionados por *immunoblotting* (245/00, M12, 94/01 e P339) e também o soro anti-PspA P278 (que apresentou baixa reatividade cruzada) foram testados quanto a sua capacidade de ligar-se à PspA presente na superfície do pneumococo intacto e

favorecer a deposição da proteína C3 do sistema complemento. Bactérias de ambos os clados foram incubadas com os diferentes soros anti-rPspAs e soro normal de camundongo, utilizado como fonte de proteínas do sistema complemento. Após incubação com anticorpo anti-C3 conjugado com FITC, a porcentagem de bactérias fluorescentes foi determinada por leitura em citômetro de fluxo e analisada com o auxílio do programa FlowJo. Foram consideradas positivas as células que apresentaram intensidade de fluorescência maior que 10. A comparação entre os histogramas de pneumococos incubados com os soros selecionados é mostrada na (Figura 10). Anticorpos gerados contra rPspA 245/00 foram capazes de induzir a deposição de complemento em 4 das 5 cepas de clado 1 testadas (setas vermelhas, coluna A) e também favoreceram a deposição de C3 na superfície de pneumococos de clado 2 (setas vermelhas, coluna C). O mesmo foi observado para anticorpos gerados contra PspA 94/01, que favoreceram a deposição de complemento em cepas de ambos os clados (Setas vermelhas coluna B e D). Anticorpos anti-PspAs M12 e P339 apresentaram reduzida capacidade de induzir a deposição de complemento em parte das cepas clado 1 e clado 2, apresentando portanto baixa reatividade cruzada. O soro anti-PspA P278, que apresentou baixa reatividade cruzada por *immunoblotting*, também apresentou capacidade reduzida de induzir a deposição de complemento em pneumococos de família 1.

4.1.4 Opsonofagocitose de pneumococos mediada por anticorpos anti-PspA

Os dois soros que apresentaram maior capacidade de favorecer a deposição de complemento na superfície de pneumococos de família 1 foram testados quanto à sua capacidade de promover a opsonização e morte por fagocitose de pneumococos por células peritoneais murinas. Os números de UFC recuperadas de cada bactéria incubada com soro anti-PspA 245/00 ou 94/01 são mostrados na Figura 11 - os dados referem-se à diluição de 1:16, com exceção da cepa P1079, em que a opsonofagocitose foi observada somente na diluição de 1:8 do soro anti-PspA 94/01.

Figura 10. Deposição da proteína C3 do sistema complemento na superfície de pneumococos de família 1 na presença dos anticorpos selecionados por *immunoblotting*. Cepas de pneumococos de clado 1 e 2 foram incubadas na presença de soro anti-PspAs recombinantes (Anti-PspA M12, 245/00, 94/01, P339 e P278). Soro de camundongos que receberam apenas $Al(OH)_3$ foi usado como controle e está representado pelas áreas cinzas. A porcentagem de bactérias fluorescentes (Intensidade de FITC > 10) foi calculada para cada amostra.

Figura 11. Ensaio de opsonofagocitose utilizando soros anti-PspAs clado 1 e 2 e células peritoneais murinas. Pneumococos de família 1 foram incubados com soro de camundongos imunizados com PspAs 245/00 ou 94/01 (Clado 1 e 2 respectivamente) e soro normal de camundongo. Os pneumococos opsonizados foram incubados com células peritoneais murinas e plaqueadas em Agar sangue. Soro de animais injetados somente com Al(OH)₃ foi utilizado como controle. Após 18 h o número de UFC recuperadas foi contado e comparados usando ANOVA e teste de Tukey. As barras representam o erro padrão da média e “*” indicam diferença estatística significativa (**p<0,001; *p<0,01).

O soro anti-PspA 245/00 foi capaz de induzir a opsonofagocitose e morte de cepas de ambos os clados, reduzindo o número de UFC recuperadas em ao menos 40% para cepas de clado 1, e 30% para cepas de clado 2. Com exceção da cepa P278, o soro anti-PspA 94/01 foi

capaz de reduzir o número de UFC recuperadas em no mínimo 30% para cepas de ambos os clados, atingindo o máximo de 46 e 63% para cepas de clado 1 e 2, respectivamente. A redução do número de UFC recuperadas de bactérias incubadas com soros anti-245/00 ou 94/01 foi estatisticamente significativa quando comparadas a bactérias incubadas com soro de animais injetados somente com $\text{Al}(\text{OH})_3$ (com exceção da cepa P278). Ambos os soros apresentaram similar capacidade de induzir a opsonofagocitose e morte dos pneumococos testados, não havendo diferença estatística entre o soro anti-PspA 245/00 e 94/01. A análise microscópica da interação entre as células peritoneais e pneumococos é mostrada na Figura 12. O grupo controle (Figura 12-A) é representado por um macrófago após incubação com pneumococo previamente incubado com soro de animais injetados somente com $\text{Al}(\text{OH})_3$. A Figura 12-B mostra a interação da célula peritoneal com pneumococos de clado 1 após opsonização com soro anti-PspA 94/01 (clado 2); e a presença de bactérias aderidas na superfície da célula indica a capacidade desse soro de induzir proteção cruzada. A internalização de pneumococos pré-opsonizados por células peritoneais é mostrada nas Figura 12-C e D, que são representativas da incubação das células com a cepa de pneumococo P69 pré-opsonizada com soro anti-PspA 245/00, ambos de clado 1.

Figura 12. Fagocitose de *S. pneumoniae* por células peritoneais murinas. **A** - Controle negativo, macrófago peritoneal incubado com pneumococo previamente incubado com soro de animal injetado com $\text{Al}(\text{OH})_3$. **B** – Macrófago peritoneal após incubação com pneumococo contendo PspA de clado 1 pré-opsonizado com soro anti-PspA 94/01 (clado 2). **C** e **D** – macrófago e neutrófilo peritoneais, respectivamente, incubados com pneumococos pré-opsonizados com antissoro anti-PspA 245/00, ambos de clado 1.

4.2 rPspA2-PdT – Obtenção da proteína híbrida e avaliação da resposta imunológica

4.2.1 Obtenção da proteína híbrida recombinante

Para obtenção da proteína híbrida os fragmentos gênicos codificantes para PspA e PdT, obtidos por PCR, foram inseridos no vetor de clonagem pGEM-T easy, fusionados com auxílio

de enzimas de restrição, clivados e inseridos no vetor de expressão pQE-30. Na primeira etapa do trabalho foi demonstrado que ambas PspAs (245/00 e 94/01) são capazes de induzir reatividade cruzada dentro da família 1. Assim, ambas moléculas poderiam ser utilizadas para obtenção da proteína híbrida. No entanto, ao iniciar a clonagem e expressão da proteína de fusão utilizando o gene codificante para a PspA 245/00 foi observado que a proteína PspA245/00-PdT era expressa de forma truncada e portanto optou-se pelo uso da PspA 94/01. Após a clonagem, a construção pQE-30-pspA94/01-pdT foi utilizada para transformação de *E. coli* competente, a expressão foi realizada em meio 2YT e a indução foi realizada com IPTG. A expressão e purificação da proteína híbrida, que a partir deste momento será denominada PspA2-PdT, presente na fração solúvel por afinidade ao Ni^{2+} foi realizada conforme descrito anteriormente. A Figura 13 mostra o SDS-PAGE da proteína híbrida PspA2-PdT; a proteína de interesse é observada com a massa molecular esperada de ~110 kDa.

A proteína PspA2-PdT foi dialisada, lavada com Triton X-114 e analisada por *immunoblotting* utilizando-se anticorpos anti-PspA2 e anti-PdT Figura 14. Pode-se observar na Figura 14-A o reconhecimento da proteína híbrida pelo anticorpo anti-PspA2, como uma banda única; o mesmo é observado na Figura 14-B em que foi utilizado anticorpo anti-PdT. Esses resultados confirmam a expressão e purificação da proteína híbrida PspA2-PdT de forma íntegra.

Figura 13. Proteína híbrida PspA2-PdT purificada. SDS-PAGE da proteína híbrida recombinante PspA2-PdT, expressa na fração solúvel em *E. coli* M15 e purificada por cromatografia de afinidade ao Ni^{2+} . O marcador de massa molecular é mostrado à esquerda (kDa).

Figura 14. Análise por *immunoblotting* da proteína híbrida recombinante PspA2-PdT purificada por afinidade ao Ni^{2+} . Amostras de rPspA2-PdT, rPspA2 e rPdT foram separadas por SDS, transferidas para membranas de PVDF e incubadas com antissoro anti-PspA2 (A) ou anti-rPdT(B). Após incubação com anti-IgG de camundongo conjugado a HRP o *immunoblotting* foi revelado com auxílio de ECL kit e fotodocumentados. A) 1 – rPspA2, 2 – rPspA2-PdT incubados com antissoro anti-PspA2. B) 1 – rPdT, 2 – rPspA2-PdT incubados com antissoro anti-PdT. O marcador de massa molecular é mostrado à esquerda (kDa).

4.2.2 Produção de anticorpos da classe IgG induzida pela imunização com a proteína híbrida PspA2-PdT

Para avaliação da resposta imunológica humoral induzida a proteína híbrida PspA2-PdT purificada e as proteínas rPspA2 e rPdT isoladamente ou coadministradas, foram utilizadas para imunização de camundongos BALB/c. Após a terceira imunização os animais foram sangrados e o soro utilizado para quantificação de anticorpos da classe IgG induzidos contra PspA ou PdT recombinantes. Não foi observada diferença significativa na produção de anticorpos IgG anti-PspA ou anti-PdT na imunização com a proteína híbrida quando comparadas com as proteínas coadministradas ou administradas individualmente (Figura 15).

Figura 15. Quantificação de anticorpos da classe IgG anti-PspA e anti-PdT. Soro dos animais imunizados com 3 doses de rPspA2, rPspA2+rPdT ou rPspA2-PdT foram testados individualmente contra rPspA2 (A) ou rPdT (B). A análise estatística foi realizada com os testes ANOVA e Tukey.

4.2.3 Ligação de anticorpos anti-PspA2-PdT à superfície de pneumococos e deposição de complemento

Foi avaliada também a capacidade dos anticorpos induzidos de se ligarem à superfície de pneumococos intactos e favorecerem a opsonização por proteína C3 do sistema complemento. Cepas de pneumococo expressando PspAs de clado 1 ou 2 foram incubadas com os soros anti-PspA2, anti-PdT, dos antígenos coadministrados ou do soro induzido contra a proteína híbrida PspA2-PdT e em seguida incubadas com anticorpos anti-IgG de camundongo conjugado a FITC. A Figura 16 mostra a porcentagem de células fluorescentes obtidas após a análise por citometria de fluxo das cepas incubadas com os diferentes soros. Pode-se observar que soro anti-PdT não induziu ligação dos anticorpos à superfície bacteriana nem deposição de complemento, mas como já demonstrado anteriormente; anticorpos anti-PspA2 administrados individualmente se ligaram à superfície de pneumococos e induziram a deposição de complemento em cepas contendo PspA dos clados 1 ou 2. Quando o PspA2 é coadministrado com o PdT, o anticorpo resultante apresenta ligação eficiente a bactérias contendo PspA de mesmo clado, mas já não tão eficiente se a bactéria contém um PspA de clado 1 (Cepas 245/00 e P69) (Figura 16 C e D). Entretanto, se o PspA encontra-se em fusão com o PdT, isto é, na forma híbrida, o antissoro se liga de forma eficiente em cepas contendo PspA de ambos os clados 1 e 2.

Figura 16. Ensaio de ligação de anticorpos na superfície do pneumococo utilizando anticorpos produzidos contra proteína híbrida. Cepas de pneumococo de PspA clado 2 (A – 94//01, B – A66.1) e clado 1 (C – 245/00 e D – P69) foram incubadas com soros dos animais imunizados com PspA2, PdT, PspA2+PdT ou PspA2-PdT seguida por incubação com anti-IgG conjugado com FITC e análise por FACS. A porcentagem de células fluorescentes positivas para a ligação de IgG na superfície do pneumococo, após incubação com anticorpos anti-IgG-FITC foi analisada com auxílio do programa FLOW Jo, considerando positivas células que apresentaram fluorescência superior a 10. A análise estatística foi realizada ANOVA e Tukey (**p<0,001, *p<0,01; *p<0,05).

No ensaio de deposição da proteína C3 do sistema complemento, as bactérias de clado 1 e 2 foram incubadas com anticorpos dos animais imunizados, soro normal de camundongo como fonte de complemento, seguida por incubação com anti-C3 conjugado e análise por FACS. Observando a mediana da fluorescência na Figura 17 A, nota-se que quando utilizada a bactéria 94/01 (mesma bactéria utilizada para amplificação do fragmento da PspA2) os soros anti-PspA, PspA+PdT e PspA-PdT induziram níveis similares de deposição complemento. Porém, quando utilizamos cepas heterólogas expressando PspAs 2 (A66.1) ou 1 (245/00 e P69) o soro anti-PspA-PdT induziu maior deposição de complemento que o soro produzido contra as proteínas coadministradas, sendo similar ao nível de deposição de complemento induzido pela imunização somente da PspA2 (Figura 17 B, C e D).

Figura 17. Deposição de complemento mediada pela imunização com a proteína PspA2-PdT. Cepas de pneumococo de PspA família 1 (Clado 2 – (A – 94/01, B – A66.1) e clado 1 (C – 245/00 e D – P69)) foram incubadas com soros dos animais imunizados com PspA2, PdT, PspA2+PdT ou PspA2-PdT e NMS seguida por incubação com anti-C3 conjugado com FITC e analisadas por FACS. A análise foi realizada com auxílio do programa FLOW Jo e os resultados comparados utilizando-se ANOVA e Tukey (** $p < 0,001$, ** $p < 0,01$; * $p < 0,05$)

4.2.4 Opsonofagocitose e morte dos pneumococos mediado por anticorpos anti-PspA2-PdT

Para o ensaio de opsonofagocitose, os antissoros foram testados quanto a sua capacidade de induzir a opsonização, fagocitose e morte dos pneumococos. Cepas de pneumococo contendo PspAs homólogos ou heterólogos, foram previamente incubadas com os soros dos animais imunizados e NMS, e adicionadas a células peritoneais de camundongos (macrófagos e neutrófilos), e então plaqueadas em ágar sangue. Quando uma cepa homóloga contendo PspA 94/01, clado 2, é incubada com os diferentes soros, não foi observado diferença entre o número de UFC recuperada dos grupos imunizados com PspA2, PspA2+PdT ou PspA2-PdT, sendo que esse resultado corrobora o resultado observado nos ensaios de ligação de anticorpos e deposição de complemento (Figura 18-A). No entanto, quando a cepa contém um PspA heterólogo, ou seja PspA clado 1, os soros dos animais imunizados com a proteína PspA2-

PdT, foram mais eficazes na indução da opsonofagocitose e morte dos pneumococos, do que o soro dos animais imunizados com as proteínas PspA2 e PdT coadministradas (Figura 18-B).

Figura 18. Ensaio de opsonofagocitose utilizando soro de camundongos imunizados com PspA e PdT. Cepas de pneumococo 94/01 (A) e 245/00 (B) contendo PspA clado 2 e 1, respectivamente, foram incubadas com soro dos animais imunizados com PspA2, PdT, PspA2+PdT ou a proteína híbrida PspA2-PdT. Os pneumococos opsonizados foram incubados com células peritoneais murinas e plaqueadas em Agar sangue. Soro de animais administrados somente com $Al(OH)_3$ foi utilizado como controle. Após 18 h o número de UFC recuperadas foi contado e comparados usando ANOVA e teste de Tukey. (** $p < 0,001$, ** $p < 0,01$; * $p < 0,05$).

4.2.5 Atividade da Ply recombinante e ensaio de hemólise

Os anticorpos gerados pela imunização com as proteínas recombinantes foram testados também quanto a sua capacidade de inibir a atividade citolítica da pneumolisina recombinante. A atividade citolítica da Ply recombinante foi testada em hemácias de carneiro e é mostrada na Figura 19. É possível observar que, conforme a Ply é diluída em PBS ocorre uma redução na absorbância obtida, que equivale à redução na hemólise das hemácias. O PBS e a proteína inespecífica (IN) utilizados como controle apresentaram baixa absorbância. A partir desse ensaio, foi determinada a concentração da Ply em aproximadamente 16 UH/mL, sendo que cada UH corresponde à quantidade necessária de Ply para a lise de 50% das hemácias utilizadas no ensaio.

Figura 19. Atividade citolítica da rPly em hemácias de carneiro. A rPly foi diluída em PBS e em seguida incubada com hemácias de carneiro. A absorbância do sobrenadante, que corresponde à hemólise das células foi mensurada a 540 nm. PBS e uma proteína recombinante inespecífica foram utilizados como controle.

Para o ensaio de inibição da atividade citolítica da Ply por anticorpos, a Ply foi incubada com soro dos animais imunizados e em seguida incubados com hemácias de carneiro. A absorbância do sobrenadante foi medida a 540 nm e comparadas entre os grupos. A hemólise induzida pelo soro de animais que receberam somente o adjuvante foi considerada como 100% e utilizada como base de cálculo para as demais. A Figura 20 mostra que o anticorpo anti-PdT inibe significativamente a atividade citolítica da Ply, essa inibição é aumentada na presença do soro de animais imunizados com as proteínas PspA2 e PdT coadministradas ou fusionadas. Anticorpos anti-PspA2 apresentaram capacidade reduzida e não significativa na inibição da atividade da Ply.

4.3 Avaliação da Resposta celular imunológica induzida pela proteína rPspA-PdT

Para avaliar a indução de resposta celular pela proteína híbrida os animais foram imunizados com 3 doses das proteínas recombinantes PspA2, PdT e PspA2-PdT administradas com Al(OH)₃, MPLA ou na ausência de adjuvantes.

Figura 20. Inibição da atividade citolítica da rPly na presença de anticorpos. Ply recombinante (8U) foi incubada com soro dos animais imunizados (1:40) e em seguida adicionada a hemácias de carneiro. A atividade hemolítica foi determinada pela absorbância do sobrenadante a 540nm. Soro de animais que receberam somente salina e $\text{Al}(\text{OH})_3$ foi utilizado como controle e considerado como 100% de hemólise. A análise estatística utilizou ANOVA e teste de Tukey. (***) $p < 0,001$, (**) $p < 0,01$; (*) $p < 0,05$).

4.3.1 Produção de anticorpos utilizando-se $\text{Al}(\text{OH})_3$, MPLA ou sem adição de adjuvantes

Primeiramente, para avaliar a imunogenicidade de cada proteína na presença ou ausência de adjuvantes, foi analisada a produção de IgG específico em cada condição. A imunização de camundongos com PspA2 na ausência de adjuvantes foi capaz de induzir níveis significantes de anticorpos, quando comparado com o grupo controle e também ao grupo que recebeu a proteína híbrida PspA2-PdT, que se mostrou pouco imunogênico nesta condição. Entretanto, a utilização de $\text{Al}(\text{OH})_3$ foi capaz de aumentar ao menos 2 vezes a produção de anticorpos pela imunização com PspA e também promover quantidades significantes de anticorpos pela imunização com PspA2-PdT, não havendo diferença significativa na produção de IgG entre a imunização com PspA2 ou PspA2-PdT. Já a utilização de MPLA como adjuvante, induziu níveis significativos de anticorpos anti-PspA nos grupos imunizados com PspA2 e com a proteína híbrida PspA2-PdT, porém em quantidades inferiores à induzida pelo $\text{Al}(\text{OH})_3$ (Figura 21– A).

A produção de anticorpos anti-PdT pela imunização com PdT ou PspA2-PdT, embora significativa quando comparada ao grupo controle, mostrou-se bastante reduzida na ausência de adjuvantes ou na presença de MPLA. Por outro lado, a utilização de $\text{Al}(\text{OH})_3$ como adjuvante mostrou-se novamente capaz de promover quantidades significativamente superiores de IgG anti-PdT, não havendo diferença entre a produção de anticorpos induzida pela imunização com PdT ou PspA2-PdT (Figura 21– B).

Figura 21. Comparação na produção de IgG induzida pelos adjuvantes Al(OH)₃, MPLA ou na ausência de adjuvantes. Camundongos BALB/c receberam 3 doses de PspA2, PdT ou PspA2-PdT na ausência de adjuvantes e adsorvidas em Al(OH)₃ ou MPLA. Após 15 dias da última imunização os animais foram sangrados e a produção de IgG específico para PspA (A) e PdT (B) foi analisada por ELISA. A análise estatística foi realizada com os testes ANOVA e Tukey (***p<0,001, **p<0,01; *p<0,05 comparados ao respectivo grupo controle, & p<0,001 – s/Adj x Al(OH)₃, # p<0,05 – Al(OH)₃ x MPLA, a p<0,001 – PspA2-PdT s/Adj x Al(OH)₃).

4.3.2 Avaliação da produção de citocinas induzida pela imunização com PspA2-PdT

Após 15 dias da última imunização, os animais foram sacrificados, os baços coletados para cultura de esplenócitos na presença dos antígenos específicos. O sobrenadante foi coletado após 48h e utilizado para avaliação de citocinas utilizando o Kit CBA Th1, Th2 e TH17.

Na ausência de adjuvantes foi observado uma produção significativa de IL-2 e IL-6 nos grupos imunizados com PspA e PdT e estimulados com os antígenos específicos, porém, não houve indução significativa da produção destas citocinas na imunização com a proteína híbrida sem adjuvante. (Figura 22 - A-1 e B-1). Nos grupos imunizados na presença de Al(OH)₃, a produção de IL-2 foi significativamente menor do que nos animais imunizados na ausência de adjuvantes, para todos os grupos (Figura 22 - A-2). Por outro lado, a utilização de hidróxido de alumínio na imunização com PspA2-PdT foi capaz de induzir um aumento significativo na produção de IL-6 quando comparada ao grupo imunizado na ausência de adjuvantes, independentemente do estímulo utilizado (Figura 22 – B2 e B1). Essa diferença não foi observada nos animais imunizados com PspA, enquanto os grupos que receberam PdT apresentaram resultado inverso, com uma maior produção de IL-6 na ausência de adjuvantes (Figura 22 – B-1 e B-2). A imunização utilizando MPLA como adjuvante, induziu níveis semelhantes de IL-2 nos grupos PspA e PdT, na presença dos antígenos específicos, e favoreceu a produção desta citocina no grupo imunizado com PspA-PdT, após o estímulo com PdT ou

com o híbrido, quando comparada a imunização na ausência de adjuvantes (Figura 22 – B3 e B1). Já a produção de IL-6 nos animais imunizados com PspA e PdT em presença de MPLA foi reduzida quando comparado com a imunização a ausência de adjuvantes, o mesmo foi observado no grupo imunizado com PspA-PdT quando comparado a imunização com $\text{Al}(\text{OH})_3$ (Figura 22 – B-3, B-1 e B2). No entanto, não houve diferença significativa na produção de IL-2 e IL-6 quando comparados os grupos imunizados com a proteína híbrida utilizando $\text{Al}(\text{OH})_3$ ou MPLA (Figura 22 – A2 e 3; B2 e 3). Não foi observada a produção das citocinas $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, IL-17, IL-4 e IL-10 (dados não mostrados).

Figura 22. Produção de IL-2 e IL-6 na ausência de adjuvantes ou na presença de $\text{Al}(\text{OH})_3$ ou MPLA. Camundongos BALB/c receberam 3 doses de PspA2, PdT ou PspA2-PdT na ausência de adjuvantes e adsorvidas em $\text{Al}(\text{OH})_3$ ou MPLA. Após 15 dias da última imunização os animais foram eutanasiados e o baço coletado para cultura de esplenócitos. As amostras foram estimuladas com 5 μg de PspA2, 5 μg de PdT ou PspA2 + PdT totalizando 10 μg de proteínas. Após 48 h o sobrenadante da cultura foi coletado e a produção de citocinas avaliadas por CBA Kit Th1, Th2, Th17. A análise estatística foi realizada com os testes ANOVA e Tukey (** $p < 0,001$, ** $p < 0,01$; * $p < 0,05$).

4.3.3 Avaliação da produção de citocinas por esplenócitos de animais imunizados após desafio

Para comparar a produção de citocinas por esplenócitos de animais imunizados antes e após o desafio, os animais imunizados com PspA, PdT ou PspA2-PdT utilizando-se apenas $\text{Al}(\text{OH})_3$ como adjuvante, foram desafiados por via intraperitoneal 15 dias após a terceira dose de imunização utilizando-se 5×10^6 UFC da cepa de pneumococo 245/00 que expressa PspA

de clado 1. Após 24 h do desafio, os animais foram eutanasiados e o baço coletado para cultura de esplenócitos, que foi realizada conforme descrito anteriormente.

As culturas de esplenócitos dos 3 grupos imunizados apresentaram significantes níveis de IL-6, sendo que os esplenócitos de animais imunizados com a proteína híbrida e estimulados com PspA produziram níveis superiores aos imunizados com a PspA2 individualmente (Figura 23 – A). No entanto, somente esplenócitos dos animais imunizados com a proteína híbrida PspA2-PdT apresentaram níveis significantes de IFN- γ e TNF- α quando estimulados com PspA e comparadas ao grupo controle, ou imunizados com PspA2 ou PdT individualmente (Figura 23 - B e C). Moderado nível de IL-17 foi observado na imunização com PspA2 e PdT, quando estimulado com as respectivas proteínas; no entanto, no grupo imunizado com PspA2-PdT, somente o estímulo com PspA foi capaz de induzir a produção de IL-17 (Figura 23– D). Uma baixa produção de IL-4 foi observada em todos os grupos imunizados (Figura 23– E). Não foram observados níveis significantes de IL-2 e IL-10 (dados não mostrados).

4.4 Avaliação do efeito protetor da rPspA2-PdT

4.4.1 Desafio fatal utilizando cepa de pneumococo com PspA heteróloga

Os animais foram desafiados após 15 dias da última dose de imunização. Foram injetadas por via intravenosa 5×10^6 UFC da cepa de pneumococo 491/00 que expressa PspA1 (heteróloga). A imunização com PspA2 foi capaz de proteger significantemente os animais quando comparada ao grupo controle e animais imunizados com PdT. O mesmo foi observado na imunização com PspA2 e PdT coadministradas ou em forma de proteína híbrida, que também promoveram proteção superior, porém não significativamente maior, que a imunização com PspA2 (Figura 24).

Figura 23. Avaliação de citocinas em animais imunizados com PspA2, PdT ou PspA2-PdT na presença de Al(OH)₃ e desafiados. Camundongos BALB/c receberam 3 doses de PspA2, PdT ou PspA2-PdT adsorvidas em Al(OH)₃. Após 15 dias da última imunização os animais foram desafiados por via intraperitoneal com 5×10^6 UFC da cepa 245/00. Os animais foram eutanasiados 24 h após o desafio e o baço coletado para cultura de esplenócitos. As amostras foram estimuladas com 5 µg de PspA2, 5 µg de PdT ou PspA2 + PdT totalizando 10 µg de proteínas. Após 48 h o sobrenadante da cultura foi coletado e a produção de citocinas avaliadas por CBA Kit Th1, Th2, Th17. A análise estatística foi realizada com os testes ANOVA e Tukey (***p<0,001, **p<0,01; *p<0,05).

Figura 24. Desafio letal por via intravenosa utilizando cepa de pneumococo com PspA heteróloga. Animais injetados com salina + Al(OH)₃ (Controle), imunizados com 3 doses de rPspA2, rPdT, rPspA2 e rPdT coadministradas (rPspA2-PdT) ou a proteína híbrida rPspA2-PdT receberam 5×10^6 UFC da cepa pneumocócica 491/00 por via intravenosa. A sobrevivência dos animais foi monitorada durante 15 dias e comparadas utilizando Mann-Whitney U test. (***p<0,001, **p<0,01; *p<0,05).

4.5 Vacinas pneumocócicas baseadas em BCG recombinante

4.5.1 Expressões de antígenos de *S. pneumoniae* em BCG

Para expressão dos antígenos de pneumococo em BCG recombinante, a fusão gênica codificante para PspA-PdT, assim como os fragmentos gênicos das proteínas SP 0148 e 2108 foram inseridos no vetor de expressão pMIP12. As construções pMIP12-*pspA2*-PdT, pMIP12-SP 0148 e pMIP12-SP 2108 foram utilizadas para transformação da cepa Pasteur de BCG. O BCG transformado foi plaqueado em meio MB7H10-OADC-Can, e as colônias resultantes foram transferidas para meio líquido para expressão das proteínas pneumocócicas, que foi posteriormente avaliada por *immunoblotting*.

Para o BCG recombinante transformado com a construção pMIP12-*pspA2*-PdT (rBCG Hib) foram testados dois clones, em sua fração solúvel e insolúvel. Podemos observar que ambos os clones foram capazes de expressar a proteína híbrida PspA2-PdT utilizando-se anticorpos anti-PspA2 (Figura 25– A) ou anti-PdT (Figura 24– B), preferencialmente na fração solúvel (colunas 2 e 3) em comparação com a fração insolúvel (colunas 5 e 6). O BCG não transformado foi utilizado como controle negativo também em sua fração solúvel e insolúvel (Figura 25 colunas 1 e 4, respectivamente), enquanto a proteína híbrida recombinante purificada de *E. coli* foi utilizada como controle positivo (Figura 25 - colunas A7 e B7).

Figura 25. Avaliação da expressão da proteína híbrida PspA-PdT em BCG recombinante por immunoblotting. Amostras de rBCG Hib lisados foram separadas por SDS-PAGE - 30 µg de cada fração solúvel (1, 2 e 3) ou 10 µg da fração insolúvel (4, 5 e 6) - e transferidas para membrana de PVDF. As membranas foram incubadas com anticorpo anti-rPspA2 (A) na diluição de 1:1000 ou anti-rPdT (B) na diluição de 1:500. Em seguida, a membrana foi incubada com anti-IgG de camundongo conjugado com HRP. A revelação foi realizada utilizando-se o kit ECL-Primer e m fotodocumentador. Frações solúveis: 1 – BCG (Controle negativo); 2 – rBCG Hib Clone 1; 3 – rBCG Hib Clone 2; Frações insolúveis – BCG (Controle negativo); 5 – rBCG Hib Clone 1; 6 – rBCG Hib Clone 2; 7 - rPspA2-PdT (Controle positivo). O marcador de massa molecular é mostrado à esquerda (kDa).

A avaliação da expressão da proteína SP 0148 em BCG utilizando-se anticorpo específico mostrou que a proteína pode ser encontrada tanto na fração solúvel (Figura 26 - coluna 2) quanto na fração insolúvel (Figura 26- coluna 3). O BCG não transformado foi

utilizado como controle negativo como fração solúvel e insolúvel (Figura 26 – colunas 1 e 3, respectivamente). A proteína recombinante foi utilizada como controle positivo (Figura 26 – coluna 5).

Figura 26. Avaliação da expressão da proteína pneumocócica SP-0148 em BCG recombinante por immunoblotting. Uma amostra de rBCG-0148 foi lisada por sonicação, separada por SDS-PAGE (30 µg da fração solúvel ou 10 µg da fração insolúvel) e transferida para membrana de PVDF. A membrana foi incubada com antissoro anti-rSP-0148 (1:1000) seguida por incubação com anti-IgG de camundongo conjugado com HRP. Frações solúveis: 1 – BCG, (Controle negativo) 2- rBCG-0148; Frações insolúveis: 3 – BCG (Controle negativo), 4 – rBCG-0148 e 5 – rSP-0148 (Controle positivo). O marcador de massa molecular é mostrado à esquerda (kDa).

Foram testados cinco clones transformados com pMIP12-SP 2108. O *immunoblotting* utilizando-se anticorpos específicos mostrou que todos os clones foram capazes de expressar a proteína pneumocócica tanto na fração solúvel (Figura 27– A, colunas 2-6) quanto na fração insolúvel (Figura 27– B, colunas 2-6). O BCG não transformado foi utilizado como controle negativo como fração solúvel e insolúvel (Figura 27, colunas 1). A proteína recombinante foi utilizada como controle positivo (Figura 27– colunas 7).

Figura 27. Avaliação da expressão da proteína pneumocócica SP-2108 em BCG recombinante por immunoblotting. Amostras de rBCG-2108 lisados foram separadas por SDS-PAGE – A) - 30 µg da fração solúvel ou B) - 10 µg do pellet - e transferidas para membrana de PVD e incubadas com anticorpo anti-rSP 2108 (1:1000), seguida por incubação com anti-IgG de camundongo conjugado com HRP. A revelação foi realizada utilizando-se o kit ECL-Primer e fotodocumentador. A) Frações solúveis: 1 – BCG (controle negativo); 2-6 – rBCG-2108 clones 1 ao 5; 7 – rSP-2108 (controle positivo). B) Frações insolúveis: 1 – Controle negativo; 2-6- rBCG-2108 clones 1 ao 5; 7 – rSP-2108 (controle positivo). O marcador de massa molecular é mostrado à esquerda (kDa).

4.5.2 Avaliação da resposta imunológica após imunização com rBCG Mix.

Em colaboração com o Professor Richard Malley os primeiros ensaios de imunização e desafio foram realizados no *Boston Children's Hospital*. Primeiramente, os animais foram imunizados com uma dose contendo 3×10^5 de cada BCG recombinante (BCG MIX), além disso foi utilizado um grupo imunizado apenas com BCG e outro com a vacina celular não encapsulada de pneumococo (WCV) como controle positivo. Não foi possível observar a indução de IL-17 na cultura de sangue total, assim como a presença de anticorpos anti-rPspA2 ou rPdT no soro. Assim, optou-se por uma dose booster contendo as proteínas recombinantes purificadas e misturadas.

Após a imunização com a dose booster de proteínas recombinantes foi possível observar um aumento significativo na indução de anticorpos anti-PspA (Figura 28-A) e anti-PdT (Figura 28-B) quando comparados ao grupo BCG controle. A dose booster de proteínas recombinantes também foi capaz de induzir a produção de IL-17 na cultura de sangue obtido do grupo imunizado com rBCG Mix quando estimulada com WCV (Figura 29-A) e níveis superiores quando estimuladas com as proteínas recombinantes mixadas (Figura 29-B) comparadas a cultura de sangue de animais que receberam somente BCG controle. Entretanto, como esperado, esses níveis permaneceram inferiores a cultura de sangue estimuladas dos animais que receberam a WCV.

No ensaio preliminar de proteção, a imunização com o rBCG Mix seguida pela dose *booster* de proteínas recombinantes demonstrou-se eficaz na proteção de camundongos contra um desafio letal de pneumonia, de forma semelhante a obtida pela imunização com WCV, quando comparada aos animais que receberam somente o BCG controle (Figura 30).

Esses resultados demonstraram um potencial uso vacinal do rBCG Mix, uma vez que foi possível observar a indução de anticorpos anti-PspA e anti-PdT, produção de IL-17 e proteção quando comparados ao grupo imunizado apenas com o BCG controle. Entretanto, novos ensaios tornaram-se necessários para avaliar o efeito dessas vacinas separadamente e também compará-las a resposta de animais imunizados com o BCG controle e a dose booster de proteínas recombinantes.

Figura 28. Produção anticorpos da classe IgG anti-PspA2 ou anti-PdT com rBCG Mix + *Booster*. Soro de animais imunizados com BCG controle ou rBCG-Mix+*Booster* foram utilizadas análise de anticorpos anti-rPspA2 (A) e anti-rPdT (B) por ELISA (RD (RD)). Os valores de anticorpos estão plotados em unidades arbitrárias (U.A) (Referencia). A análise estatística realizada por Mann–Whitney U test. (***) $p < 0,001$

Figura 29. Produção IL-17 em cultura de sangue total após a imunização com rBCG Mix + *Booster*. Amostras de sangue heparinizados de animais imunizados com WCV, BCG controle ou rBCG-Mix+*Booster* foram utilizadas para cultura de sangue total utilizando 5 μ g de WCV (A) ou 5 μ g de mix das proteínas recombinantes (B) como estímulos. A presença de IL-17 no sobrenadante foi avaliada após 6 dias por ELISA (RD) e análise estatística realizada comparando-se o grupo rBCG-Mix+*Booster* ao grupo BCG controle por Mann–Whitney U test.

Figura 30. Desafio letal por pneumonia após imunização com rBCG Mix+Booster. Animais imunizados com WCV, BCG controle ou rBCG-Mix+Booster foram desafiados por via intranasal com 1×10^6 da cepa pneumocócica WU2. A sobrevivência dos animais foi monitorada durante 15 dias e comparada ao grupo controle BCG+Booster utilizando Mann–Whitney U test.

4.5.3 Avaliação da resposta imunológica das vacinas rBCG-0148, rBCG-2108, rBCG Hib separadamente e rBCG Mix

A fim de avaliar a resposta imunológica induzida por cada uma das vacinas de rBCG separadamente, camundongos C57BL/6 foram imunizados com 1×10^6 de rBCG-0148, rBCG-2108 e rBCG-PspA-PdT ou ainda com rBCG Mix. Animais imunizados com 1×10^6 de BCG controle ou injetados com solução salina foram utilizados como controle. Após 36 dias da imunização, os grupos de animais imunizados, com exceção do grupo injetado com solução salina, receberam uma dose *booster* com as respectivas proteínas recombinantes. Os grupos BCG controle e rBCG Mix receberam as proteínas recombinantes misturadas.

4.5.3.1 Avaliação de anticorpos pela imunização com rBCG e booster de proteínas recombinantes

A presença de anticorpos induzidos pela imunização contra as proteínas rSP-0148, rSP-2108, rPspA2, rPdT foi avaliada no soro 15 dias após a dose *booster* de proteínas. Foi observado um aumento na produção de anticorpos contra rPspA2 em animais imunizados com o rBCG Hib após o *booster* com a proteína rPspA2-PdT, entretanto, a mesma produção foi observada em animais que receberam somente o BCG controle+Booster, assim como nos animais que receberam o rBCG Mix+Booster (Figura 31-A). Resultado semelhante foi

observado no soro de animais imunizados com rBCG-2108+*Booster*, quando comparados aos grupos BCG controle+*Booster* e BCG Mix+*Booster* (Figura 31-D). A imunização rBCG Mix seguida pelo *booster* com rPspA2-PdT foi capaz de induzir níveis significantes de anticorpos anti-rPdT quando comparada a imunização com o BCG controle+*Booster* (Figura 31-B). Não foi observada produção significativa de anticorpos entre os grupos BCG Controle+*Booster*, rBCG-0148+*Booster* e rBCG Mix+*Booster*. (Figura 31-C). Nenhuma produção de anticorpos foi observada nos grupos imunizados somente com os rBCG antes da dose *booster* de proteínas recombinantes (Figura 31)

4.5.3.2 Avaliação de citocinas em cultura de esplenócitos após imunização com rBCG e booster de proteínas recombinantes

A fim de avaliar a indução de citocinas pela imunização com os rBCG foram realizadas cultura de esplenócitos 21 dias após a dose *booster* com proteínas recombinantes. A presença de citocinas no sobrenadante foi avaliada utilizando-se Kit CBA (BD Bioscience). A cultura de esplenócitos de animais imunizados rBCG-0148+*Booster* foi capaz de induzir níveis significantes de IL-17 quando estimulada com a respectiva proteína recombinante e superiores a produção de IL-17 observada nos grupos rBCG+ *Booster* e rBCG Mix+*Booster* após estímulo com a mesma proteína (Figura 32-A). Resultado semelhante foi observado para a produção de IFN- γ . (Figura 32-B). Não foi observado produção significativa dessas citocinas no grupo imunizado com rBCG Mix+*Booster* quando comparado ao grupo rBCG+*Booster*. Não foi observada a produção de IL-2, IL-4, IL-6, TNF- α e IL-10.

Figura 31. Produção de anticorpos da classe IgG pela imunização com os rBCG+Booster. Os animais foram imunizados com uma dose de rBCG seguida por uma dose *booster* de proteínas recombinantes. A presença de anticorpos contra cada proteína foi avaliada no soro por ELISA. A) Anticorpos anti-PspA2; B) Anticorpos anti-PdT; C) Anticorpos anti-rSP 0148 e D) IgG anti-rSP 2108. Foi utilizado IgG de camundongo como curva padrão. Foram utilizados 10 animais por grupo. As barras representam desvio padrão e a análise estatística foi realizada com os testes ANOVA e Tukey (** $p < 0,01$).

A cultura de esplenócitos de animais imunizados com rBCG-2108+Booster e rBCG-Mix+Booster apresentaram níveis significativos de IL-17 e IFN- γ (Figura 33-A e B, respectivamente) quando estimuladas com rSP 2108 e comparados com a mesma amostra não estimulada. Entretanto, não foi observado diferenças significativas na comparação nos níveis de citocinas produzidos pela cultura de esplenócitos dos animais imunizados com BCG+Booster e estimuladas com a mesma proteína (Figura 33). Não foi observada a produção de IL-2, IL-4, IL-6, TNF- α e IL-10, assim como a presença das citocinas testadas nas culturas

de esplenócitos de animais imunizados com rBCG Hib+*Booster* estimuladas com rPspa2 ou rPdT.

Figura 32. Produção IL-17 e IFN- γ pela imunização com rBCG 0148+*Booster* em resposta ao estímulo rSP-0148. Os esplenócitos de animais que receberam salina + Al(OH)₃ ou imunizados com BCG Controle+*Booster*, rBCG-0148+*Booster* e rBCG-Mix+*Booster* foram cultivados na ausência de estímulos (NE) ou na presença de 5 μ g rSP-0148 (SP 0148), a presença de citocinas no sobrenadante foi analisada após 48 h por CBA TH1 TH2 Th17 (BD Bioscience). A) Produção de IL-17; B) Produção de IFN- γ . Foram utilizados 10 animais por grupo. As barras representam o desvio padrão. A análise estatística foi realizada com os testes ANOVA e Tukey (** p <0,001; * p <0,01; * p <0,05).

Figura 33. Produção IL-17 e IFN- γ pela imunização com rBCG 2108+*Booster* em resposta ao estímulo rSP-2108. Os esplenócitos de animais que receberam salina + Al(OH)₃ ou foram imunizados com BCG Controle+*Booster*, rBCG-2108+*Booster* e rBCG-Mix+*Booster* foram cultivados na ausência de estímulos (NE) ou na presença de 5 μ g de rSP-2108 (SP 2108), a presença de citocinas no sobrenadante foi analisada após 48 h por CBA Th1, Th2, Th17 (BD Bioscience). A) Produção de IL-17; B) Produção de IFN- γ . Foram utilizados 10 animais por grupo. As barras representam o desvio padrão. E a análise estatística foi realizada com os testes ANOVA e Tukey (** p <0,001, * p <0,01; * p <0,05).

4.5.4 Avaliação da proteção induzida pela imunização com rBCG + *Booster*

A capacidade dos rBCG em induzirem proteção contra desafio letal e colonização foi avaliada 21 dias após a dose *booster* de proteínas recombinantes.

4.5.4.1 Avaliação da proteção contra desafio letal de pneumonia

Os animais anestesiados receberam 1×10^7 UFC da cepa pneumocócica WU2 por via intranasal. A sobrevivência dos animais foi observada durante 15 dias. A imunização com rBCG-0148+*Booster* e rBCG-2108+*Booster* não foi capaz de proteger os camundongos neste modelo de desafio, apresentando níveis de sobrevivência similares ao grupo controle que recebeu apenas salina e hidróxido de alumínio (Figura 34). Os grupos imunizados com rBCG Hib+ *Booster* e rBCG Mix+*Booster* mostraram níveis de sobrevivência superiores aos grupos mencionados anteriormente, entretanto, não foi possível observar diferença na sobrevivência de camundongos quando comparados ao grupo BCG Controle que também recebeu uma dose das proteínas recombinantes (Figura 34).

4.5.4.2 Avaliação da proteção contra colonização

Para avaliação da proteção contra colonização pneumocócica os animais imunizados foram desafiados por via intranasal com a cepa 603 e o lavado traqueonasal realizado após 7 dias. O número de UFC recuperadas foi contado após plaqueamento em ágar sangue. Foi possível observar redução, embora não significativa, no número de UFC recuperadas no lavado nasal dos animais imunizados com rBCG-0148+*Booster* quando comparados aos animais do grupo salina, BCG +*Booster*, rBCG-2108+*Booster* e rBCG Hib+*Booster* (Figura 35). Interessantemente, os animais que receberam rBCG-Mix+*Booster* apresentaram redução significativa no número de UFC quando comparados aos demais grupos (Figura 35).

5 DISCUSSÃO

Diversas proteínas têm sido investigadas como alternativa para vacinas pneumocócicas, visando induzir ampla proteção contra os diferentes sorotipos de pneumococo e também memória imunológica. A imunização com a PspA e a pneumolisina têm mostrado efeitos protetores em diferentes combinações e modelos animais (BRILES et al., 2003; DANIELS et al., 2010; DENOEL et al., 2011).

A PspA é considerada um importante candidato vacinal contra doenças pneumocócicas; entretanto, sua variabilidade estrutural e sorológica pode limitar a cobertura de uma vacina baseada nesta proteína. Alguns trabalhos têm demonstrado que o nível de reatividade cruzada e proteção cruzada entre PspAs correlaciona-se com a similaridade entre suas sequências, sendo baixa entre PspAs de diferentes famílias e alta entre PspAs de mesma família. Entretanto, tem sido sugerido que o nível de reatividade e proteção cruzadas sejam variáveis dependendo do clado da PspA (DARRIEUX et al., 2008; NABORS et al., 2000).

No presente estudo, foi analisado o nível de reatividade cruzada entre PspAs da família 1 (clados 1 e 2). Foram produzidas 10 proteínas recombinantes contendo a região N-terminal de 5 PspAs de clado 1 e 5 PspAs de clado 2, que foram avaliadas quanto à indução de anticorpos capazes de reagir cruzadamente com PspAs da mesma família. Observou-se que, enquanto anticorpos induzidos por imunização com algumas rPspAs, por exemplo P13 e 373/00, foram capazes de reconhecer apenas PspAs extraídas de pneumococos de mesmo clado, quatro deles – anti-PspA 245/00, M12, 94/01 e P339 – apresentaram reatividade cruzada com extratos obtidos de pneumococos de ambos os clados. Resultados semelhantes foram obtidos em outros trabalhos que utilizaram análises por *immunoblotting*, onde foi observada alta heterogeneidade no nível de reatividade cruzada induzida por diferentes rPspAs (DARRIEUX et al., 2008; NABORS et al., 2000). Embora todos os fragmentos de PspA produzidos incluam as regiões A, B e o início da região rica em prolinas, alguns contêm a região de prolina completa, incluindo a região conhecida como Non-Pro (uma região que codifica uma sequência não rica em prolinas no meio de duas sequências ricas em prolinas). Essa diferença no tamanho das proteínas deve-se ao alinhamento do oligonucleotídeo reverso no momento da PCR - como a região rica em prolina é bastante repetitiva, o alinhamento do oligonucleotídeo pode ocorrer em regiões aleatórias. Entretanto, não foi observada por *immunoblotting* uma relação clara entre o tamanho do fragmento e o nível de reatividade cruzada induzida, já que 3 dos fragmentos que apresentaram maior reatividade cruzada são longos e um (M12) é curto, como pode ser visto na Figura 9. Nos ensaios funcionais como deposição de complemento e opsonofagocitose, os

anticorpos que apresentaram ampla reatividade cruzada foram gerados pela imunização de animais com os fragmentos proteicos mais longos e contendo a região rica em prolina completa, incluindo o bloco Non-Pro. Vadesilho e colaboradores, demonstraram recentemente que a proteção contra pneumococo é mediada por anticorpos conformacionais, presentes principalmente nas regiões entre os aminoácidos 29 e 238, não havendo proteção quando utilizados peptídeos sintéticos (VADESILHO et al., 2014). Além disso, foi demonstrado que a imunização de camundongos com a região Non-Pro é capaz de induzir proteção contra desafio fatal com pneumococo (DANIELS et al., 2010). Esses dados sugerem que a utilização de moléculas mais longas pode induzir anticorpos de maior reatividade e que inclusão do bloco Non-Pro possa participar desta reatividade cruzada.

A fagocitose dependente de anticorpos opsonizantes é considerada o principal mecanismo de morte do pneumococo. A capacidade dos anticorpos anti-PspAs de promover a deposição de complemento na superfície da bactéria contribui para o efeito protetor da proteína (BROWN; HOSEA; FRANK, 1983). Entretanto, tem sido demonstrado que o nível de deposição de complemento depende da similaridade entre a PspA usada para induzir os anticorpos e a PspA expressa pelo pneumococo (DARRIEUX et al., 2008; REN et al., 2004). Para avaliar essa importante propriedade funcional, quatro soros selecionados na análise por *immunoblotting*, foram testados quanto à capacidade de induzir a deposição de complemento em diversas cepas de pneumococo. A análise por citometria de fluxo demonstrou que os anticorpos anti-PspA 245/00 e anti-PspA 94/01 foram capazes de induzir melhor deposição da proteína C3 do sistema complemento no maior número de cepas testadas, quando comparados com os soros anti-PspA M12 e anti-PspA P339. O soro anti-PspA P278, que apresentou baixa reatividade cruzada por *immunoblotting*, apresentou também pouca capacidade de induzir a deposição de complemento em diferentes cepas. A deposição de complemento em diferentes pneumococos parece ser influenciada pelo sorotipo da cápsula bacteriana. Durante o estudo foi observado que alguns sorotipos apresentam aumento na deposição de complemento mesmo na ausência de anticorpos anti-PspA, como demonstrado previamente com o sorotipo 6B (MELIN et al., 2009).

A virulência de cepas de *S. pneumoniae* em camundongos se restringe a um pequeno número de sorotipos (BENTON; PATON; BRILES, 1997; CHIAVOLINI; POZZI; RICCI, 2008), dificultando a realização de ensaios de desafio e proteção, principalmente quando há a necessidade da utilização de um número significativo de cepas para testar a capacidade de uma proteína em induzir anticorpos com ampla reatividade cruzada. Para avaliar a capacidade dos anticorpos induzidos contra as proteínas recombinantes de reagir cruzadamente com PspAs

expressas em pneumococos de diferentes sorotipos, foi adaptado um protocolo de ensaio de opsonofagocitose (OPA). Esse ensaio é comumente utilizado para avaliar a proteção de anticorpos induzidos contra polissacarídeos de *S. pneumoniae*, utilizando a linhagem celular HL-60 (STEINER, 1999) e até o momento, não estava padronizado para avaliação de anticorpos gerados contra proteínas. O protocolo de opsonofagocitose adaptado utiliza fagócitos peritoneais de camundongos BALB/c previamente estimulados com ConA. Além de recrutar fagócitos para a cavidade peritoneal, a administração dessa proteína estimula o espraio de macrófagos, a fagocitose e produção de H_2O_2 (RODRIGUES et al., 2002).

Os ensaios de opsonofagocitose induzida por anticorpos gerados contra as PspAs 245/00 e 94/01, utilizando 8 cepas de pneumococos de diferentes sorotipos, demonstraram que ambos os soros reduziram significativamente o número de UFC recuperadas de cada cepa, quando comparados ao grupo controle. Esse resultado mostra que os anticorpos induzidos pelas PspA 245/00 e 94/01 reagem cruzadamente dentro família 1 e são capazes de inibir a ação desta molécula na superfície da bactéria, favorecendo a morte de pneumococos por opsonofagocitose de forma independente do sorotipo capsular, indicando um potencial protetor. Este foi publicado (GOULART et al., 2011-Apêndice VII). Recentemente, novas adaptações demonstraram o uso deste ensaio para avaliação de anticorpos anti-PspA, demonstrando a necessidade de buscar novas ferramentas para estudo e validação de novas vacinas. No entanto, estes trabalhos utilizaram soro de humanos imunizados com PspA ou anticorpos monoclonais e células HL-60 ou neutrófilos purificados de sangue periférico humano, respectivamente (DANIELS et al., 2013; GENSCHEMER; ACCAVITTI-LOPER; BRILES, 2013).

A fusão de proteínas de pneumococo tem sido utilizada com o intuito de ampliar e melhorar a resposta imunológica. Uma construção contendo PsaA fusionada a PdT foi capaz de induzir anticorpos para ambas as proteínas, além de atuar eficientemente como proteína carreadora quando conjugada ao polissacarídeo C de pneumococo (LU et al., 2009). Nguyen e colaboradores, demonstraram que a fusão de PspA com flagelina foi capaz de aumentar a proteção contra desafios invasivos (NGUYEN et al., 2011). Baseado nesses resultados, a segunda etapa do projeto visou obter uma proteína de fusão contendo a porção N-terminal da PspA 94/01 (PspA2) fusionada à PdT, uma forma geneticamente detoxificada da Ply (PATON et al., 1991). A imunização de camundongos com a proteína híbrida rPspA2-PdT, induziu níveis similares de anticorpos contra PspA e PdT, quando comparados aos níveis de anticorpos produzidos pela imunização com as proteínas administradas isoladamente ou coadministradas, demonstrando que não há predominância imunogênica.

A Ply não está acessível a anticorpos na superfície do pneumococo (BERRY et al., 1989; PATON, 2011), portanto, os testes *in-vitro* em que avaliamos a funcionalidade dos anticorpos induzidos pela imunização, ou seja, a capacidade de se ligarem na superfície do pneumococo, favorecendo a opsonização e morte por fagocitose, são válidos somente para os anticorpos anti-PspA. A análise da capacidade desses anticorpos de se ligarem em PspAs expressas na superfície de diferentes cepas de pneumococo, demonstrou inicialmente que a coadministração de PspA2 e PdT não perturba a produção de anticorpos de ligação eficiente no caso de cepas contendo PspA de mesmo clado, mas diminui a sua eficiência em relação a cepas contendo PspA de clado diferente. Esse efeito é revertido quando a PspA2 se encontra em fusão com o PdT, na forma de proteína híbrida, quando os anticorpos produzidos apresentam alta eficiência de ligação tanto a cepas contendo PspA de clado 1 quanto de clado 2.

Quando avaliada a indução de deposição de complemento, a mesma tendência é observada e os anticorpos produzidos contra as proteínas coadministradas apresentaram a mesma atividade de deposição de complemento que os anticorpos gerados contra a proteína híbrida PspA2-PdT na cepa homóloga 94/01. Estes mesmos anticorpos se mostraram mais eficazes na indução da deposição de complemento nas outras cepas testadas de ambos os clados.

Os resultados obtidos nos ensaios de OPA estão de acordo com o observado nos na ligação de anticorpos e deposição de complemento, onde novamente foi observado que os soros anti-rPspA2, coadministrados ou híbrido PspA2-PdT promoveram a opsonofagocitose e morte de pneumococos que possuem PspA homóloga à utilizada na proteína híbrida. Enquanto que utilizando uma cepa de pneumococo de PspA clado 1, foi observado uma capacidade significativamente maior na promoção da opsonização, fagocitose e morte de pneumococos induzida por anticorpos dos animais que receberam a proteína híbrida ou PspA2 administrada individualmente, quando comparados com os anticorpos induzidos pela coadministração das proteínas.

Embora, não tenha sido observada diferença nos níveis de anticorpos, hipotetizamos que a coadministração ou fusão das proteínas, possa influenciar na afinidade dos anticorpos que é observada quando os soros são testados contra a proteína em sua conformação nativa na superfície do pneumococo. Esses resultados assemelham-se aos observados na conjugação de uma PspA de clado 1 com o polissacarídeo 6B, onde, embora o processo de conjugação tenha promovido uma redução de cerca de 20% da estrutura alfa-hélice da PspA, os anticorpos induzidos pela imunização com os conjugados promoveram uma maior opsonofagocitose da cepa de pneumococo testada, de forma independente do polissacarídeo capsular, que os anticorpos induzidos contra a PspA coadministrada com o PS (PERCIANI et al., 2013). Além

disso, foi observado que a adição do adjuvante MF-59 nas vacinas contra vírus de influenza H1N1 e H5N1 foi capaz de melhorar quantitativa e qualitativamente a resposta de anticorpos funcionais, ampliando o repertório de epítomos reconhecidos por células B e ainda melhorando sua afinidade dos anticorpos produzidos (KHURANA et al., 2010; KHURANA et al., 2011; O'HAGAN et al., 2011). Assim, devido as propriedades adjuvantes do PdT, que pode atuar de forma sinérgica com TLR4 ou ainda promover maior ativação de células dendríticas e produção de anticorpos contra um antígeno específico independentemente da interação com TLR (MALLEY et al., 2003; MCNEELA et al., 2010), sua fusão com a PspA pode ter promovido uma melhora qualitativa na resposta funcional dos anticorpos, ampliando a reatividade cruzada entre PspAs heterólogas.

A proteção mediada pela imunização com pneumolisóides está associada a capacidade dos anticorpos de neutralizar a ação citotóxica da Ply liberada durante a infecção (PATON; LOCK; HANSMAN, 1983). O ensaio de inibição de hemólise tem sido utilizado a fim de avaliar a capacidade dos anticorpos em neutralizar a ação da Ply; os resultados demonstram que a fusão das proteínas PspA2-PdT mantém a capacidade de gerar anticorpos com essa função, assim como a PdT administrada individualmente ou em combinação com a PspA2. Embora os soros produzidos contra a rPspA2 tenham apresentado uma sutil inibição da hemólise, esse resultado não é significativo e também não está relacionado com similaridade nas sequências de aminoácidos entre as duas proteínas, entretanto pode influenciar na maior inibição da hemólise na combinação e na fusão das proteínas (GOULART et al. 2013-Apêndice I).

A utilização de adjuvantes tem como objetivo promover ou melhorar a resposta imunológica induzida por vacinas de subunidades ou DNA. A escolha do adjuvante deve ser baseada no tipo de resposta necessária para proteção contra o patógeno estudado. A proteção contra pneumococos foi, por muito tempo, descrita como mediada por anticorpos opsonizantes, direcionados não apenas contra a cápsula polissacarídica, mas também contra proteínas. Entretanto, estudos recentes têm demonstrado a importância de uma boa resposta celular especialmente durante a colonização (BASSET et al., 2007; COHEN et al., 2011; MALLEY et al., 2005). Nós observamos que a imunização com PspA2 na ausência de adjuvantes foi capaz de induzir significantes níveis de anticorpos. Entretanto, a utilização de $Al(OH)_3$ na imunização com a proteína híbrida mostrou-se mais eficaz na produção de anticorpos, principal característica do adjuvante. O MPLA tem como principal característica estimular a resposta inata, uma vez que atua como um padrão molecular associado ao patógeno (PAMP) e interage com TLR4, tendo levado a uma indução reduzida de anticorpos (Revisado em (MOHAN; VERMA; RAO, 2013; SIVAKUMAR et al., 2011)).

A infecção por pneumococo leva à produção de diversas citocinas que atuam no recrutamento de células para o local da infecção, levando à indução de uma resposta imune inata e também auxiliando no desencadeamento de uma resposta imunológica adquirida, que em conjunto levariam à eliminação da bactéria (JOYCE; POPPER; FALKOW, 2009; PAATS et al., 2013). A indução de resposta celular mediada pela produção de citocinas tem sido bastante estudada, especialmente em modelos de proteção contra colonização por pneumococo (COHEN et al., 2011; LIMA et al., 2012; LU et al., 2008). Recentemente, foi demonstrado que a produção de IFN- γ induz proteção contra infecção pneumocócica por inibir a progressão da bactéria presente no pulmão para uma doença invasiva (LEMESSURIER, 2013). A IL-2 é produzida por células T ativadas, atua como fator de crescimento de células B estimulando a síntese de anticorpos, já a IL-6 é um membro da família de citocinas do tipo 6 e tem um papel fundamental tanto na resposta inata e ativação de neutrófilos, quanto na resposta imune adquirida, onde estimula células B a se desenvolverem em plasmócitos produtores de anticorpos (AKDIS et al., 2011).

A imunização com a proteína recombinante PspA2-PdT utilizando-se $\text{Al}(\text{OH})_3$ ou MPLA foi capaz de induzir níveis significantes de IL-2 e IL-6, sendo que a utilização de hidróxido de alumínio induziu uma produção superior de IL-6 quando comparado ao MPLA. Foi demonstrado que a infecção de camundongos com uma cepa de pneumococo expressando Ply estimula maior produção de IL-6 que a mesma cepa deletada deste gene (BENTON; EVERSON; BRILES, 1995). Assim, a produção aumentada de IL-6 observada nas imunizações com a proteína híbrida, pode estar relacionada com a presença do PdT fusionado a PspA. A presença destas citocinas é importante pois demonstra que as células estão ativadas e são capazes de gerar uma resposta frente aos antígenos específicos (AKDIS et al., 2011). Neste modelo não foram observadas citocinas características de uma polarização da resposta celular em Th1, Th2 ou Th17. Com base em outros estudos utilizando proteínas pneumocócicas, foi realizado um ensaio de resposta celular onde os animais foram imunizados utilizando-se $\text{Al}(\text{OH})_3$ como adjuvante, desafiados e após 24 h os esplenócitos submetidos a cultura. A escolha do $\text{Al}(\text{OH})_3$ para este experimento, deve-se ao fato do uso deste adjuvante no ensaio anterior, ter apresentado maior indução de anticorpos anti-PspA e anti-PdT, e também ter apresentado uma maior indução de IL-6, que embora não seja estatisticamente significativa é aproximadamente 2 vezes superior à produção induzida pelo uso de MPLA. Neste modelo, podemos observar um aumento na produção de IL-6 pelos grupos imunizados, sendo que, a produção desta citocina pelo grupo imunizado com rPspA2-PdT foi significantemente superior ao grupo imunizado com rPspA2 e também estimulado com o mesmo antígeno. A imunização

com a proteína híbrida mostrou-se também mais eficaz na produção de IFN- γ e TNF- α , já que somente os grupos que receberam rPspA2-PdT foram capazes de produzir níveis significantes destas citocinas, quando estimuladas com rPspA2. Lima e colaboradores, demonstraram que a cultura de células do lavado bronqueoalveolar de animais desafiados com pneumococo, e estimuladas com PspA, apresentaram níveis significativos de IL-6, IFN- γ e TNF- α nas primeiras h após o desafio, sendo que o controle desta inflamação, observado pela redução destas citocinas após 24 h, em cultura de células provenientes de animais previamente imunizados com PspA e vacina celular de pertussis, um potente adjuvante, está diretamente relacionado com a proteção dos animais imunizado (LIMA et al., 2012). Esses resultados sugerem que a imunização com a fusão da PspA com o PdT foi capaz de promover uma maior indução de resposta celular, quando comparada a imunização com os antígenos individualmente, e pode promover uma resposta efetora superior frente ao desafio pneumocócico.

A IL-17 tem demonstrado importante papel na proteção contra patógenos como *Bordetella pertussis* e *Mycobacterium tuberculosis* (HIGGINS et al., 2006; KHADER et al., 2007), assim como na proteção contra a colonização por pneumococos (COHEN et al., 2011). Malley e colaboradores demonstraram que a imunização com uma vacina celular de pneumococo, não encapsulado e inativado, é capaz de induzir proteção contra colonização, mediada por células T CD4⁺ produtoras de IL-17, independentemente da produção de anticorpos (MALLEY et al., 2006). Nós observamos que a imunização com rPspA2 ou rPdT foi capaz de produzir IL-17 quando estimulada com os respectivos antígenos, entretanto, a imunização rPspA2-PdT somente produziu essa citocina quando estimulada com rPspA2. Também foi observado uma produção reduzida de IL-4.

Diferentes modelos de desafio têm sido utilizados no estudo de antígenos vacinais como colonização, pneumonia lobar, sepse por desafio intranasal, intraperitoneal ou intravenoso. A proteção induzida pelas proteínas recombinantes foi testada por desafio intravenoso utilizando uma cepa que expressa PspA de clado 1, onde a imunização com rPspA2, proteínas coadministradas ou fusionadas aumentaram significativamente a sobrevivência dos animais. O fato de não observarmos proteção pela imunização com a rPdT pode ser devido ao modelo utilizado; a proteção mediada por Ply é comumente testada por desafio intranasal, não sendo relatado até o momento proteção por sepse intravenosa, um método bastante utilizado para teste de proteção mediada pela imunização com PspAs. Assim, neste modelo também não foi possível observar o efeito protetor aditivo mediado pela combinação ou fusão das proteínas.

A vacina de BCG é a única disponível contra o agente causador da tuberculose *Mycobacterium tuberculosis*. Devido a suas propriedades adjuvantes, esta vacina tem sido estudada como vetor de expressão para antígenos heterólogos, a fim de induzir uma resposta imunológica contra ambos os agentes. Langermann et al., 1994, demonstrou que a expressão de rPspA em BCG recombinante foi capaz de proteger camundongos contra desafio letal. Assim, em parceria com o professor Dr. Richard Malley (*Division of Infectious Diseases, Department of Medicine, Children's Hospital, and Harvard Medical School, Boston, MA*) foram desenvolvidas vacinas pneumocócicas utilizando-se o BCG como vetor de expressão. Nós obtivemos com sucesso a expressão da proteína híbrida PspA2-PdT, bem como outras duas proteínas denominadas SP 0148 e SP 2108. As proteínas pneumocócicas foram encontradas tanto na porção solúvel, que é constituída de proteínas presentes no citosol, quanto na porção insolúvel do lisado celular, que é constituído por proteínas associadas à parede e membrana celular, e também por células intactas. Antígenos associados a membrana do BCG tendem a ser mais imunogênicos que os localizados no citoplasma (BASTOS et al., 2002), no entanto ensaios de localização subcelular precisam ser realizados para confirmar estes dados. A associação das proteínas à parede celular do BCG poderia promover uma resposta semelhante à induzida pelas proteínas SP 0148 e SP 2108 purificadas, onde a lipidação destas, e consequente interação com TLR2, mostrou ser essencial para a proteção (MOFFITT et al., 2014).

Ao analisarmos a resposta imunológica induzida por essas vacinas observamos que a imunização com apenas uma dose dos BCG recombinantes não foi suficiente para a geração de anticorpos específicos anti-PspA e anti-PdT - necessários para indução de proteção contra desafio letal (REN et al., 2004; SWIATLO et al., 2003), e também insuficiente para indução de IL-17 - a principal citocina envolvida na proteção contra colonização (MOFFITT et al., 2011). Resultados semelhantes já foram observados onde a imunização com rBCG não foi capaz de induzir anticorpos específicos contra as proteínas heterólogas (NASCIMENTO et al., 2008; VARALDO et al., 2004). Assim, adotamos a estratégia de *prime-boost*, onde os animais receberam uma dose de BCG recombinante seguida por uma dose das respectivas proteínas.

A imunização com rBCG Hib utilizando a estratégia de *prime-boost* foi capaz de induzir anticorpos anti-rPspA e anti-rPdT. Entretanto, não foi observada diferença significativa nos níveis de anticorpos quando comparada ao grupo controle BCG+Booster. PspA é uma proteína altamente imunogênica e a quantidade utilizada de proteína recombinante pode gerar anticorpos específicos mesmo no grupo que foi primado apenas com o BCG controle. Resultado semelhante foi observado nos anticorpos induzidos contra rPdT, onde apenas o grupo rBCG Mix+Booster, que recebeu uma dose reduzida de rBCG Hib (3×10^5 UFC) como primer foi

capaz de induzir um aumento significativo de anticorpos quando comparados ao BCG+*Booster*. Os grupos imunizados com rBCG Hib e rBCG Mix que receberam o *booster* de proteínas recombinantes contendo rPspA2-PdT foram capazes de aumentar a sobrevivência dos animais após o desafio letal. No entanto, não houve diferença significativa quando comparada ao grupo BCG controle que também recebeu o *booster* contendo rPspA2-PdT. Uma vez que a proteção pela imunização com PspA é mediada pela presença de anticorpos capazes de neutralização a ação da PspA e levar a opsonização da bactéria (REN et al., 2004; TU et al., 1999), e a utilização de pneumolisóides leva a produção de anticorpos capazes de inibir a atividade citolítica da pneumolisina (PATON; LOCK; HANSMAN, 1983; SALHA et al., 2012), o resultado obtido no deasfio letal está de acordo com o observado na produção de anticorpos anti-PspA e anti-PdT, onde não houve diferença na proteção induzida pelos grupos que receberam a dose *booster*. Estes resultados sugerem a necessidade da otimização da dose de proteína recombinante utilizada para a dose *booster* ou ainda da dose primária de rBCG.

A imunização com o BCG-0148 seguida pela dose *booster* de proteínas recombinantes, mostrou-se eficaz na indução de IL-17 e IFN- γ na cultura de esplenócitos e foi suficiente para a redução do número de UFC recuperados após a colonização da nasofaringe quando comparados ao grupo controle BCG+*booster*. Entretanto, não induziu anticorpos específicos e proteção contra desafio letal. Esses resultados estão de acordo com os dados da literatura onde a imunização com a SP-0148 é capaz de induzir proteção contra colonização mediada pela presença de IL-17. (MOFFITT et al., 2011). Enquanto, o grupo imunizado com rBCG-2108, não mostrou diferença significativa na indução de anticorpos, assim como na produção de IL-17. Consequentemente, esta imunização falhou na proteção de camundongos nos dois modelos avaliados. Ao compararmos as construções rBCG-0148 e rBCG-2108 por *immunoblotting* podemos observar que a expressão da SP 0148 é muito mais acentuada que a SP 2108, podendo estar relacionado a indução de uma resposta imunológica diferenciada.

A combinação dos BCG recombinantes obtidos mostrou-se mais eficiente na proteção contra colonização, apresentando uma redução significativa de UFC recuperadas o lavado nasal quando comparada aos demais grupos. Entretanto, analisando a produção das citocinas IL-17 e IFN- γ em cultura de esplenócitos não foram observadas diferenças significativas entre o grupo imunizado com rBCG Mix+*Booster* e BCG+*Booster* diante dos estímulos SP 0148 e SP 2108. Este resultado sugere um efeito sinérgico induzido pela combinação dos rBCG utilizados, porém, torna-se necessário avaliar outros parâmetros como a presença de citocinas no lavado nasal e também a presença de anticorpos da classe IgA que possam estar colaborando na redução de UFC e ainda a necessidade de avaliar diferentes combinações a fim de definir se

este efeito é causado pela combinação das três vacinas ou pode ser induzido pela combinação da rBCG-0148 com rBCG-2108 ou rBCG Hib. O efeito sinérgico das proteínas SP 0148 e SP 2108 vêm sendo avaliado em ensaio clínico em combinação com uma terceira proteína hipotética, os resultados da primeira fase mostraram a necessidade de 3 doses contendo 100 µg das proteínas misturadas para a indução de anticorpos e IL-17 (GENOCEA, 2014). Estes resultados sugerem um uso potencial da vacina de BCG como vector de expressão para proteínas pneumocócicas, podendo levar a redução da colonização da nasofaringe, fase inicial no desenvolvimento de doenças pneumocócicas. Além disso, o uso da estratégia de *primer-boost* pode levar a redução de doses vacinais necessárias para indução de resposta imunológica e proteção.

6 CONCLUSÕES

Nossos resultados demonstram que moléculas de PspA de mesmo clado ou família podem induzir diferentes níveis de reatividade cruzada. Assim, foi possível selecionar 2 moléculas de PspA, capazes de induzir anticorpos com ampla reatividade cruzada, isoladas das cepas de *Pneumococo* 245/00 e 94/01, PspA de clado 1 e 2, respectivamente, sugerindo que a inclusão de uma dessas moléculas em uma formulação vacinal deve promover proteção contra infecção por cepas de pneumococos de família 1. Também, foi possível padronizar um ensaio de opsonofagocitose utilizando-se soro anti-PspA recombinante que poderá auxiliar no estudo de outros antígenos vacinais.

A fusão da PspA2 com PdT resultou numa proteína híbrida rPspA2-PdT que manteve suas propriedades imunogênicas, capaz de induzir a opsonofagocitose de cepas de pneumococos de mesmo clado e também capaz de ampliar ainda mais a reatividade cruzada dentro da família 1 por induzir anticorpos com maior afinidade a cepas contendo PspAs heterólogos de clado 1, promover maior opsonização por deposição da proteína C3 do sistema complemento e consequentemente induzir a opsonofagocitose quando comparada às proteínas coadministradas. Portanto, sugerimos que em uma formulação vacinal, baseada na combinação de proteínas de superfície e citosólicas, os potenciais candidatos vacinais PspA e pneumolisóides sejam administrados na forma de proteínas de fusão, a fim de assegurar uma maior proteção por reatividade cruzada entre as PspAs.

O uso de vacina de BCG como vetor de expressão para proteínas pneumocócicas, utilizando-se a estratégia de *primer-booster*, mostrou-se eficiente na indução de IL-17 nos animais imunizados com rBCG-0148. Este grupo também apresentou eficaz redução do número de UFC recuperadas após desafio de colonização. Interessantemente, a combinação de rBCG-0148, rBCG-2108 e rBCG Hib na imunização de camundongos, foi capaz de induzir proteção significativa contra colonização, quando comparado aos demais grupos imunizados, sugerindo um efeito sinérgico dessas proteínas que necessita ser esclarecido. Assim, sugerimos que BCG recombinante expressando proteínas pneumocócicas pode ser utilizado como potencial candidato vacinal contra colonização pneumocócica.

REFERÊNCIAS*

- ABEYTA, M.; HARDY, G. G.; YOTHER, J. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. **Infect. Immun.**, v. 71, n. 1, p. 218-225, 2003.
- AFONSO, E. T.; MINAMISAVA, R.; BIERRENBACH, A. L.; ESCALANTE, J. J.; ALENCAR, A. P.; DOMINGUES, C. M.; MORAIS-NETO, O. L.; TOSCANO, C. M.; ANDRADE, A. L. Effect of 10-valent pneumococcal vaccine on pneumonia among children, Brazil. **Emerging Infectious Diseases**, v. 19, n. 4, p. 589-597, 2013.
- AKDIS, M.; BURGLER, S.; CRAMERI, R.; EIWEGGER, T.; FUJITA, H.; GOMEZ, E.; KLUNKER, S.; MEYER, N.; O'MAHONY, L.; PALOMARES, O.; RHYNER, C.; OUAKED, N.; SCHAFFARTZIK, A.; VAN DE VEEN, W.; ZELLER, S.; ZIMMERMANN, M.; AKDIS, C. A. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. **The Journal of Allergy and Clinical Immunology**, v. 127, n. 3, p. 701-721 e701-770, 2011.
- ALCANTARA, R. B.; PREHEIM, L. C.; GENTRY-NIELSEN, M. J. Pneumolysin-induced complement depletion during experimental pneumococcal bacteremia. **Infect. Immun.**, v. 69, n. 6, p. 3569-3575, 2001.
- ALDOVINI, A.; YOUNG, R. A. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. **Nature**, v. 351, n. 6326, p. 479-482, 1991.
- ALEXANDER, J. E.; LOCK, R. A.; PEETERS, C. C.; POOLMAN, J. T.; ANDREW, P. W.; MITCHELL, T. J.; HANSMAN, D.; PATON, J. C. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. **Infect. Immun.**, v. 62, n. 12, p. 5683-5688, 1994.
- AMI, Y.; IZUMI, Y.; MATSUO, K.; SOMEYA, K.; KANEKIYO, M.; HORIBATA, S.; YOSHINO, N.; SAKAI, K.; SHINOHARA, K.; MATSUMOTO, S.; YAMADA, T.; YAMAZAKI, S.; YAMAMOTO, N.; HONDA, M. Priming-boosting vaccination with recombinant Mycobacterium bovis bacillus Calmette-Guerin and a nonreplicating vaccinia virus recombinant leads to long-lasting and effective immunity. **Journal of Virology**, v. 79, n. 20, p. 12871-12879, 2005.
- ANDRADE, A. L.; TERNES, Y. M.; VIEIRA, M. A.; MOREIRA, W. G.; LAMARO-CARDOSO, J.; KIPNIS, A.; CARDOSO, M. R.; BRANDILEONE, M. C.; MOURA, I.; PIMENTA, F. C.; DA GLORIA CARVALHO, M.; SARAIVA, F. O.; TOSCANO, C. M.; MINAMISAVA, R. Direct effect of 10-valent conjugate pneumococcal vaccination on pneumococcal carriage in children Brazil. **PLoS One**, v. 9, n. 6, p. e98128, 2014.

*De acordo com:

ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. **NBR 6023**: informação e documentação: referências: elaboração. Rio de Janeiro, 2002.

ANDRADE, P. M.; CHADE, D. C.; BORRA, R. C.; NASCIMENTO, I. P.; VILLANOVA, F. E.; LEITE, L. C.; ANDRADE, E.; SROUGI, M. The therapeutic potential of recombinant BCG expressing the antigen S1PT in the intravesical treatment of bladder cancer. **Urologic Oncology**, v. 28, n. 5, p. 520-525, 2010.

ARAMA, C.; WASEEM, S.; FERNANDEZ, C.; ASSEFAW-REDDA, Y.; YOU, L.; RODRIGUEZ, A.; RADOSEVIC, K.; GOUDSMIT, J.; KAUFMANN, S. H.; REECE, S. T.; TROYE-BLOMBERG, M. A recombinant Bacille Calmette-Guerin construct expressing the Plasmodium falciparum circumsporozoite protein enhances dendritic cell activation and primes for circumsporozoite-specific memory cells in BALB/c mice. **Vaccine**, v. 30, n. 37, p. 5578-5584, 2012.

ARULANANDAM, B. P.; LYNCH, J. M.; BRILES, D. E.; HOLLINGSHEAD, S.; METZGER, D. W. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. **Infect. Immun.**, v. 69, n. 11, p. 6718-6724, 2001.

ATTALI, C.; FROLET, C.; DURMORT, C.; OFFANT, J.; VERNET, T.; DI GUILMI, A. M. *Streptococcus pneumoniae* choline-binding protein E interaction with plasminogen/plasmin stimulates migration across the extracellular matrix. **Infect. Immun.**, v. 76, n. 2, p. 466-476, 2008a.

ATTALI, C.; DURMORT, C.; VERNET, T.; DI GUILMI, A. M. The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. **Infect. Immun.**, v. 76, n. 11, p. 5350-5356, 2008b.

AUSTRIAN, R.; GOLD, J. Pneumococcal bacteremia with special reference to bacteremic pneumococcal pneumonia. **Ann. Internal. Med.**, v. 60, n., p. 759-776, 1964.

BALACHANDRAN, P.; HOLLINGSHEAD, S. K.; PATON, J. C.; BRILES, D. E. The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. **J. Bacteriol.**, v. 183, n. 10, p. 3108-3116, 2001.

BASSET, A.; THOMPSON, C. M.; HOLLINGSHEAD, S. K.; BRILES, D. E.; ADES, E. W.; LIPSITCH, M.; MALLEY, R. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. **Infect. Immun.**, v. 75, n. 11, p. 5460-5464, 2007.

BASTOS, R. G.; DELLAGOSTIN, O. A.; BARLETTA, R. G.; DOSTER, A. R.; NELSON, E.; OSORIO, F. A. Construction and immunogenicity of recombinant Mycobacterium bovis BCG expressing GP5 and M protein of porcine reproductive respiratory syndrome virus. **Vaccine**, v. 21, n. 1-2, p. 21-29, 2002.

BAYLOR, N. W.; EGAN, W.; RICHMAN, P. Aluminum salts in vaccines--US perspective. **Vaccine**, v. 20, p. S18-23, 2002. Suppl 3.

BEALL, B.; GHERARDI, G.; FACKLAM, R. R.; HOLLINGSHEAD, S. K. Pneumococcal pspA sequence types of prevalent multiresistant pneumococcal strains in the United States and of internationally disseminated clones. **J. Clin. Microbiol.**, v. 38, n. 10, p. 3663-3669, 2000.

BEHR, M. A. BCG--different strains, different vaccines? **The Lancet Infectious Diseases**, v. 2, n. 2, p. 86-92, 2002.

BEN-SHIMOL, S.; GREENBERG, D.; GIVON-LAVI, N.; SCHLESINGER, Y.; SOMEKH, E.; AVINER, S.; MIRON, D.; DAGAN, R. Early impact of sequential introduction of 7-valent and 13-valent pneumococcal conjugate vaccine on IPD in Israeli children <5 years: an active prospective nationwide surveillance. **Vaccine**, v. 32, n. 27, p. 3452-3459, 2014.

BENTON, K. A.; EVERSON, M. P.; BRILES, D. E. A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice. **Infect. Immun.**, v. 63, n. 2, p. 448-455, 1995.

BENTON, K. A.; PATON, J. C.; BRILES, D. E. Differences in virulence for mice among *Streptococcus pneumoniae* strains of capsular types 2, 3, 4, 5, and 6 are not attributable to differences in pneumolysin production. **Infect. Immun.**, v. 65, n. 4, p. 1237-1244, 1997.

BENTON, K. A.; VANCOTT, J. L.; BRILES, D. E. Role of tumor necrosis factor alpha in the host response of mice to bacteremia caused by pneumolysin-deficient *Streptococcus pneumoniae*. **Infect. Immun.**, v. 66, n. 2, p. 839-842, 1998.

BERGLUND, J.; VINK, P.; TAVARES DA SILVA, F.; LESTRATE, P.; BOUTRIAU, D. Safety, immunogenicity, and antibody persistence following an investigational *Streptococcus pneumoniae* and *Haemophilus influenzae* triple-protein vaccine in a phase 1 randomized controlled study in healthy adults. **Clin. Vaccine Immunol.**, v. 21, n. 1, p. 56-65, 2014.

BERGMANN, S.; HAMMERSCHMIDT, S. Versatility of pneumococcal surface proteins. **Microbiology**, v. 152, pt. 2, p. 295-303, 2006.

BERRY, A. M.; LOCK, R. A.; HANSMAN, D.; PATON, J. C. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. **Infect. Immun.**, v. 57, n. 8, p. 2324-2330, 1989.

BERRY, A. M.; ALEXANDER, J. E.; MITCHELL, T. J.; ANDREW, P. W.; HANSMAN, D.; PATON, J. C. Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*. **Infect. Immun.**, v. 63, n. 5, p. 1969-1974, 1995.

BOGAERT, D.; DE GROOT, R.; HERMANS, P. W. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. **The Lancet Infectious Diseases**, v. 4, n. 3, p. 144-154, 2004.

BOLOGA, M.; KAMTCHOUA, T.; HOPFER, R.; SHENG, X.; HICKS, B.; BIXLER, G.; HOU, V.; PEHLIC, V.; YUAN, T.; GURUNATHAN, S. Safety and immunogenicity of pneumococcal protein vaccine candidates: monovalent choline-binding protein A (PcpA) vaccine and bivalent PcpA-pneumococcal histidine triad protein D vaccine. **Vaccine**, v. 30, n. 52, p. 7461-7468, 2012.

BRANDILEONE, M. C.; DE ANDRADE, A. L.; DI FABIO, J. L.; GUERRA, M. L.; AUSTRIAN, R. Appropriateness of a pneumococcal conjugate vaccine in Brazil: potential impact of age and clinical diagnosis, with emphasis on meningitis. **J. Infect. Dis.**, v. 187, n. 8, p. 1206-1212, 2003.

BRANDILEONE, M. C.; ANDRADE, A. L.; TELES, E. M.; ZANELLA, R. C.; YARA, T. I.; DI FABIO, J. L.; HOLLINGSHEAD, S. K. Typing of pneumococcal surface protein A (PspA) in *Streptococcus pneumoniae* isolated during epidemiological surveillance in Brazil: towards novel pneumococcal protein vaccines. **Vaccine**, v. 22, n. 29-30, p. 3890-3896, 2004.

BRASIL. **Ministério da saúde do brasil**. Proposta para introdução da vacina pneumocócica 10-valente (conjugada) no calendário básico de vacinação da criança: incorporação Março-2010. Disponível em: http://portal.saude.gov.br/portal/arquivos/pdf/intro_pneumococica10_val_04_02_10_ver_final.pdf. Acesso em: 15, Ago, 2013.

BRILES, D. E.; KING, J. D.; GRAY, M. A.; MCDANIEL, L. S.; SWIATLO, E.; BENTON, K. A. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. **Vaccine**, v. 14, n. 9, p. 858-867, 1996.

BRILES, D. E.; HOLLINGSHEAD, S.; BROOKS-WALTER, A.; NABORS, G. S.; FERGUSON, L.; SCHILLING, M.; GRAVENSTEIN, S.; BRAUN, P.; KING, J.; SWIFT, A. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. **Vaccine**, v. 18, n. 16, p. 1707-1711, 2000a.

BRILES, D. E.; HOLLINGSHEAD, S. K.; KING, J.; SWIFT, A.; BRAUN, P. A.; PARK, M. K.; FERGUSON, L. M.; NAHM, M. H.; NABORS, G. S. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. **J. Infect. Dis.** v. 182, n. 6, p. 1694-1701, 2000b.

BRILES, D. E.; HOLLINGSHEAD, S. K.; PATON, J. C.; ADES, E. W.; NOVAK, L.; VAN GINKEL, F. W.; BENJAMIN, W. H., JR. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. **J. Infect. Dis.**, v. 188, n. 3, p. 339-348, 2003.

BROOKS-WALTER, A.; BRILES, D. E.; HOLLINGSHEAD, S. K. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. **Infect. Immun.**, v. 67, n. 12, p. 6533-6542, 1999.

BROWN, E. J., S. W. HOSEA, AND M. M. FRANK. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. **Rev. Infect. Dis.**, v. 5(Suppl. 4), n., p. S797-S805., 1983.

BROWN, J. S.; OGUNNIYI, A. D.; WOODROW, M. C.; HOLDEN, D. W.; PATON, J. C. Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. **Infect. Immun.**, v. 69, n. 11, p. 6702-6706, 2001.

BURNAUGH, A. M.; FRANTZ, L. J.; KING, S. J. Growth of *Streptococcus pneumoniae* on human glycoconjugates is dependent upon the sequential activity of bacterial exoglycosidases. **J. Bacteriol.**, v. 190, n. 1, p. 221-230, 2008.

CALIX, J. J.; NAHM, M. H. A new pneumococcal serotype, 11E, has a variably inactivated *wcjE* gene. **J. Infect. Dis.**, v. 202, n. 1, p. 29-38, 2010.

CARDOZO, D. M.; NASCIMENTO-CARVALHO, C. M.; ANDRADE, A. L.; SILVANY-NETO, A. M.; DALTRO, C. H.; BRANDAO, M. A.; BRANDAO, A. P.; BRANDILEONE, M. C. Prevalence and risk factors for nasopharyngeal carriage of *Streptococcus pneumoniae* among adolescents. **J. Med. Microbiol.**, v. 57, n. Pt 2, p. 185-189, 2008.

CAYABYAB, M. J.; KORIOTH-SCHMITZ, B.; SUN, Y.; CARVILLE, A.; BALACHANDRAN, H.; MIURA, A.; CARLSON, K. R.; BUZBY, A. P.; HAYNES, B. F.; JACOBS, W. R.; LETVIN, N. L. Recombinant *Mycobacterium bovis* BCG prime-recombinant adenovirus boost vaccination in rhesus monkeys elicits robust polyfunctional simian immunodeficiency virus-specific T-cell responses. **Journal of Virology**, v. 83, n. 11, p. 5505-5513, 2009.

CHANDLER, L. J.; REISNER, B. S.; WOODS, G. L.; JAFRI, A. K. Comparison of four methods for identifying *Streptococcus pneumoniae*. **Diagnostic Microbiology and Infectious Disease**, v. 37, n. 4, p. 285-287, 2000.

CHAPMAN, R.; CHEGE, G.; SHEPHARD, E.; STUTZ, H.; WILLIAMSON, A. L. Recombinant *Mycobacterium bovis* BCG as an HIV vaccine vector. **Current HIV Research**, v. 8, n. 4, p. 282-298, 2010.

CHIAVOLINI, D.; POZZI, G.; RICCI, S. Animal models of *Streptococcus pneumoniae* disease. **Clin. Microbiol. Rev.**, v. 21, n. 4, p. 666-685, 2008.

COHEN, J. M.; KHANDAVILLI, S.; CAMBERLEIN, E.; HYAMS, C.; BAXENDALE, H. E.; BROWN, J. S. Protective contributions against invasive *Streptococcus pneumoniae* pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. **PLoS One**, v. 6, n. 10, p. e25558, 2011.

CONNELL, N. D.; MEDINA-ACOSTA, E.; MCMASTER, W. R.; BLOOM, B. R.; RUSSELL, D. G. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. **Proc. Natl. Acad. Sci. U S A**, v. 90, n. 24, p. 11473-11477, 1993.

CONRADT, P.; HESS, J.; KAUFMANN, S. H. Cytolytic T-cell responses to human dendritic cells and macrophages infected with *Mycobacterium bovis* BCG and recombinant BCG secreting listeriolysin. **Microbes Infect.**, v. 1, n. 10, p. 753-764, 1999.

CUNDELL, D. R.; PEARCE, B. J.; SANDROS, J.; NAUGHTON, A. M.; MASURE, H. R. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. **Infect. Immun.**, v. 63, n. 7, p. 2493-2498, 1995a.

CUNDELL, D. R.; GERARD, N. P.; GERARD, C.; IDANPAAN-HEIKKILA, I.; TUOMANEN, E. I. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. **Nature**, v. 377, n. 6548, p. 435-438, 1995b.

DAGAN, R.; MELAMED, R.; MUALLEM, M.; PIGLANSKY, L.; YAGUPSKY, P. Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. **J. Infect. Dis.**, v. 174, n. 6, p. 1352-1355, 1996.

DANIELS, C. C.; COAN, P.; KING, J.; HALE, J.; BENTON, K. A.; BRILES, D. E.; HOLLINGSHEAD, S. K. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. **Infect. Immun.**, 2010.

DANIELS, C. C.; KIM, K. H.; BURTON, R. L.; MIRZA, S.; WALKER, M.; KING, J.; HALE, Y.; COAN, P.; RHEE, D. K.; NAHM, M. H.; BRILES, D. E. Modified opsonization, phagocytosis, and killing assays to measure potentially protective antibodies against pneumococcal surface protein A. **Clin. Vaccine Immunol.**, v. 20, n. 10, p. 1549-1558, 2013.

DARRIEUX, M.; MIYAJI, E. N.; FERREIRA, D. M.; LOPES, L. M.; LOPES, A. P.; REN, B.; BRILES, D. E.; HOLLINGSHEAD, S. K.; LEITE, L. C. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. **Infect. Immun.**, v. 75, n. 12, p. 5930-5938, 2007.

DARRIEUX, M.; MORENO, A. T.; FERREIRA, D. M.; PIMENTA, F. C.; DE ANDRADE, A. L.; LOPES, A. P.; LEITE, L. C.; MIYAJI, E. N. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. **J. Med. Microbiol.**, v. 57, n. Pt 3, p. 273-278, 2008.

DAVIS, K. M.; AKINBI, H. T.; STANDISH, A. J.; WEISER, J. N. Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by *Streptococcus pneumoniae*. **PLoS pathogens**, v. 4, n. 12, p. e1000241, 2008.

DENOEL, P.; PHILIPP, M. T.; DOYLE, L.; MARTIN, D.; CARLETTI, G.; POOLMAN, J. T. A protein-based pneumococcal vaccine protects rhesus macaques from pneumonia after experimental infection with *Streptococcus pneumoniae*. **Vaccine**, v. 29, n. 33, p. 5495-5501, 2011.

DESEL, C.; DORHOI, A.; BANDERMANN, S.; GRODE, L.; EISELE, B.; KAUFMANN, S. H. Recombinant BCG DeltaureC hly+ induces superior protection over parental BCG by stimulating a balanced combination of type 1 and type 17 cytokine responses. **J. Infect. Dis.**, v. 204, n. 10, p. 1573-1584, 2011.

DOWSON, C. What is a pneumococcus? In: TUOMANEN, E. I.; MITCHELL, T. J.; DONALD, A. M.; BRIAN, G. S. (Ed.). **The pneumococcus**. Washington, D.C.: ASM Press, 2004. p. 3-14.

ELM, C.; BRAATHEN, R.; BERGMANN, S.; FRANK, R.; VAERMAN, J. P.; KAETZEL, C. S.; CHHATWAL, G. S.; JOHANSEN, F. E.; HAMMERSCHMIDT, S. Ectodomains 3 and 4 of human polymeric Immunoglobulin receptor (hIgR) mediate invasion of *Streptococcus pneumoniae* into the epithelium. **The Journal of Biological Chemistry**, v. 279, n. 8, p. 6296-6304, 2004.

FEIKIN, D. R.; KAGUCIA, E. W.; LOO, J. D.; LINK-GELLES, R.; PUHAN, M. A.; CHERIAN, T.; LEVINE, O. S.; WHITNEY, C. G.; O'BRIEN, K. L.; MOORE, M. R. Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine

introduction: a pooled analysis of multiple surveillance sites. **PLoS Medicine**, v. 10, n. 9, p. e1001517, 2013.

FELDMAN, C.; MITCHELL, T. J.; ANDREW, P. W.; BOULNOIS, G. J.; READ, R. C.; TODD, H. C.; COLE, P. J.; WILSON, R. The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium in vitro. **Microb. Pathog.**, v. 9, n. 4, p. 275-284, 1990.

FELDMAN, C.; MUNRO, N. C.; JEFFERY, P. K.; MITCHELL, T. J.; ANDREW, P. W.; BOULNOIS, G. J.; GUERREIRO, D.; ROHDE, J. A.; TODD, H. C.; COLE, P. J.; ET AL. Pneumolysin induces the salient histologic features of pneumococcal infection in the rat lung in vivo. **Am. J. Respir. Cell Mol. Biol.**, v. 5, n. 5, p. 416-423, 1991.

FELDMAN, C.; ANDERSON, R.; COCKERAN, R.; MITCHELL, T.; COLE, P.; WILSON, R. The effects of pneumolysin and hydrogen peroxide, alone and in combination, on human ciliated epithelium in vitro. **Respiratory Medicine**, v. 96, n. 8, p. 580-585, 2002.

FERREIRA, D. M.; DARRIEUX, M.; OLIVEIRA, M. L.; LEITE, L. C.; MIYAJI, E. N. Optimized immune response elicited by a DNA vaccine expressing pneumococcal surface protein a is characterized by a balanced immunoglobulin G1 (IgG1)/IgG2a ratio and proinflammatory cytokine production. **Clin. Vaccine Immunol.**, v. 15, n. 3, p. 499-505, 2008.

FERREIRA, D. M.; OLIVEIRA, M. L.; MORENO, A. T.; HO, P. L.; BRILES, D. E.; MIYAJI, E. N. Protection against nasal colonization with *Streptococcus pneumoniae* by parenteral immunization with a DNA vaccine encoding PspA (Pneumococcal surface protein A). **Microb. Pathog.**, v. 48, n. 6, p. 205-213, 2010.

FRENCK, R. JR.; THOMPSON, A.; YEH, S.H.; LONDON, A.; SIDHU M.S.; PATTERSON S.; GRUBER W.C.; EMINI E.A.; SCOTT D.A.; GURTMAN A. Immunogenicity and safety of 13-valent pneumococcal conjugate vaccine in children previously immunized with 7-valent pneumococcal conjugate vaccine. **Pediatr. Infect. Dis. J.** v. 30, n. 12 p. 1086-1091, 2011

GENOCEA. GEN-004 FOR PNEUMOCOCCUS. v. n. p. 2014. Disponível em: <<http://www.genocsa.com/platform-pipeline/pipeline/gen004-for-pneumococcus/>>. Acesso em: 28, nov. 2014.

GENSCHMER, K. R.; ACCAVITTI-LOPER, M. A.; BRILES, D. E. A modified surface killing assay (MSKA) as a functional in vitro assay for identifying protective antibodies against pneumococcal surface protein A (PspA). **Vaccine**, v. 32, n. 1, p. 39-47, 2013.

GHAFFAR, F.; BARTON, T.; LOZANO, J.; MUNIZ, L. S.; HICKS, P.; GAN, V.; AHMAD, N.; MCCracken, G. H., JR. Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. **Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America**, v. 39, n. 7, p. 930-938, 2004.

GILBERT, R.J. Cholesterol-dependent cytolysins. **Adv. Exp. Med. Biol.**, v. 677 p. 56-66, 2010.

GLENNY, A. T. Insoluble precipitates in diphtheria and tetanus immunization. **British Medical Journal**, v. 2, n. 3632, p. 244-245, 1930.

GOR, D. O.; DING, X.; BRILES, D. E.; JACOBS, M. R.; GREENSPAN, N. S. Relationship between surface accessibility for PpmA, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae*. **Infect. Immun.**, v. 73, n. 3, p. 1304-1312, 2005.

GOULART, C.; DARRIEUX, M.; RODRIGUEZ, D.; PIMENTA, F. C.; BRANDILEONE, M. C.; DE ANDRADE, A. L.; LEITE, L. C. Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. **Vaccine**, v. 29, n. 8, p. 1634-1642, 2011.

GRODE, L.; GANOZA, C. A.; BROHM, C.; WEINER, J., 3RD; EISELE, B.; KAUFMANN, S. H. Safety and immunogenicity of the recombinant BCG vaccine VPM1002 in a phase 1 open-label randomized clinical trial. **Vaccine**, v. 31, n. 9, p. 1340-1348, 2013.

HAMEL, J.; CHARLAND, N.; PINEAU, I.; OUELLET, C.; RIOUX, S.; MARTIN, D.; BRODEUR, B.R. Prevention of Pneumococcal Disease in Mice Immunized with Conserved Surface-Accessible Proteins. **Infection and Immunity**, p. 2659–2670, 2004.

HANSEN, J.; BLACK, S.; SHINEFIELD, H.; CHERIAN, T.; BENSON, J.; FIREMAN, B.; LEWIS, E.; RAY, P.; LEE, J. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than 5 years of age for prevention of pneumonia: updated analysis using World Health Organization standardized interpretation of chest radiographs. **The Pediatric Infectious Disease Journal**, v. 25, n. 9, p. 779-781, 2006.

HENRIQUES-NORMARK, B.; TUOMANEN, E. I. The pneumococcus: epidemiology, microbiology, and pathogenesis. **Cold. Spring Harb. Perspect. Med.**, v. 3, p. a010215, 2013.

HICKS, L. A.; HARRISON, L. H.; FLANNERY, B.; HADLER, J. L.; SCHAFFNER, W.; CRAIG, A. S.; JACKSON, D.; THOMAS, A.; BEALL, B.; LYNFIELD, R.; REINGOLD, A.; FARLEY, M. M.; WHITNEY, C. G. Incidence of Pneumococcal Disease Due to Non-Pneumococcal Conjugate Vaccine (PCV7) Serotypes in the United States during the Era of Widespread PCV7 Vaccination, 1998-2004. **J. Infect. Dis.**, v. 196, n. 9, p. 1346-1354, 2007.

HIGGINS, S. C.; JARNICKI, A. G.; LAVELLE, E. C.; MILLS, K. H. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. **J. Immunol.**, v. 177, n. 11, p. 7980-7989, 2006.

HOLLINGSHEAD, S. K.; BECKER, R.; BRILES, D. E. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. **Infect. Immun.**, v. 68, n. 10, p. 5889-5900, 2000.

HSU, K. K.; SHEA, K. M.; STEVENSON, A. E.; PELTON, S. I. Changing serotypes causing childhood invasive pneumococcal disease: Massachusetts, 2001-2007. **The Pediatric Infectious Disease Journal**, v. 29, n. 4, p. 289-293, 2010.

JEDRZEJAS, M. J.; LAMANI, E.; BECKER, R. S. Characterization of selected strains of pneumococcal surface protein A. **The Journal of Biological Chemistry**, v. 276, n. 35, p. 33121-33128, 2001.

JOHNSON, H. L.; DELORIA-KNOLL, M.; LEVINE, O. S.; STOSZEK, S. K.; FREIMANIS HANCE, L.; REITHINGER, R.; MUENZ, L. R.; O'BRIEN, K. L. Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. **PLoS Medicine**, v. 7, n. 10, p., 2010.

JOHNSTON, R. B., JR. The host response to invasion by *Streptococcus pneumoniae*: protection and the pathogenesis to tissue damage. **Rev. Infect. Dis.**, v. 3, n. 2, p. 282-288, 1981.

JOYCE, E. A.; POPPER, S. J.; FALKOW, S. *Streptococcus pneumoniae* nasopharyngeal colonization induces type I interferons and interferon-induced gene expression. **BMC Genomics**, v. 10, n., p. 404, 2009.

KADIOGLU, A.; WEISER, J. N.; PATON, J. C.; ANDREW, P. W. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. **Nature Reviews Microbiology**, v. 6, n. 4, p. 288-301, 2008.

KAETZEL, C. S. Polymeric Ig receptor: defender of the fort or Trojan horse? **Current Biology : CB**, v. 11, n. 1, p. R35-38, 2001.

KAMTCHOUA, T.; BOLOGA, M.; HOPFER, R.; NEVEU, D.; HU, B.; SHENG, X.; CORDE, N.; POUZET, C.; ZIMMERMANN, G.; GURUNATHAN, S. Safety and immunogenicity of the pneumococcal pneumolysin derivative PlyD1 in a single-antigen protein vaccine candidate in adults. **Vaccine**, v. 31, n. 2, p. 327-333, 2013.

KAUFMANN, S. H.; COTTON, M. F.; EISELE, B.; GENGENBACHER, M.; GRODE, L.; HESSELING, A. C.; WALZL, G. The BCG replacement vaccine VPM1002: from drawing board to clinical trial. **Expert Rev. Vaccines**, v. 13, n. 5, p. 619-630, 2014.

KHADER, S. A.; BELL, G. K.; PEARL, J. E.; FOUNTAIN, J. J.; RANGEL-MORENO, J.; CILLEY, G. E.; SHEN, F.; EATON, S. M.; GAFFEN, S. L.; SWAIN, S. L.; LOCKSLEY, R. M.; HAYNES, L.; RANDALL, T. D.; COOPER, A. M. IL-23 and IL-17 in the establishment of protective pulmonary CD4⁺ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. **Nature Immunology**, v. 8, n. 4, p. 369-377, 2007.

KHURANA, S.; CHEARWAE, W.; CASTELLINO, F.; MANISCHEWITZ, J.; KING, L. R.; HONORKIEWICZ, A.; ROCK, M. T.; EDWARDS, K. M.; DEL GIUDICE, G.; RAPPUOLI, R.; GOLDING, H. Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. **Science Translational Medicine**, v. 2, n. 15, p. 15ra15, 2010.

KHURANA, S.; VERMA, N.; YEWDELL, J. W.; HILBERT, A. K.; CASTELLINO, F.; LATTANZI, M.; DEL GIUDICE, G.; RAPPUOLI, R.; GOLDING, H. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. **Science Translational Medicine**, v. 3, n. 85, p. 85ra48, 2011.

KHURANA, S.; COYLE, E. M.; DIMITROVA, M.; CASTELLINO, F.; NICHOLSON, K.; DEL GIUDICE, G.; GOLDING, H. Heterologous prime-boost vaccination with MF59-adjuvanted H5 vaccines promotes antibody affinity maturation towards the hemagglutinin HA1 domain and broad H5N1 cross-clade neutralization. **PLoS One**, v. 9, n. 4, p. e95496, 2014.

KING, S. J.; HIPPE, K. R.; WEISER, J. N. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. **Mol. Microbiol.**, v. 59, n. 3, p. 961-974, 2006.

KIRKHAM, L. A.; KERR, A. R.; DOUCE, G. R.; PATERSON, G. K.; DILTS, D. A.; LIU, D. F.; MITCHELL, T. J. Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. **Infect. Immun.**, v. 74, n. 1, p. 586-593, 2006.

KONO, M.; HOTOMI, M.; HOLLINGSHEAD, S. K.; BRILES, D. E.; YAMANAKA, N. Maternal immunization with pneumococcal surface protein A protects against pneumococcal infections among derived offspring. **PLoS One**, v. 6, n. 10, p. e27102, 2011.

KOSKELA, M.; LEINONEN, M.; HAIVA, V. M.; TIMONEN, M.; MAKELA, P. H. First and second dose antibody responses to pneumococcal polysaccharide vaccine in infants. **Pediatr. Infect. Dis.**, v. 5, n. 1, p. 45-50, 1986.

LALOR, M. K.; SMITH, S. G.; FLOYD, S.; GORAK-STOLINSKA, P.; WEIR, R. E.; BLITZ, R.; BRANSON, K.; FINE, P. E.; DOCKRELL, H. M. Complex cytokine profiles induced by BCG vaccination in UK infants. **Vaccine**, v. 28, n. 6, p. 1635-1641, 2010.

LANGERMANN, S.; PALASZYNSKI, S. R.; BURLEIN, J. E.; KOENIG, S.; HANSON, M. S.; BRILES, D. E.; STOVER, C. K. Protective humoral response against pneumococcal infection in mice elicited by recombinant bacille Calmette-Guerin vaccines expressing pneumococcal surface protein A. **J. Exp. Med.**, v. 180, n. 6, p. 2277-2286, 1994.

LEE, C. J.; LOCK, R. A.; ANDREW, P. W.; MITCHELL, T. J.; HANSMAN, D.; PATON, J. C. Protection of infant mice from challenge with *Streptococcus pneumoniae* type 19F by immunization with a type 19F polysaccharide--pneumolysoid conjugate. **Vaccine**, v. 12, n. 10, p. 875-878, 1994.

LEHMANN, D.; WILLIS, J.; MOORE, H. C.; GIELE, C.; MURPHY, D.; KEIL, A. D.; HARRISON, C.; BAYLEY, K.; WATSON, M.; RICHMOND, P. The changing epidemiology of invasive pneumococcal disease in aboriginal and non-aboriginal western Australians from 1997 through 2007 and emergence of nonvaccine serotypes. **Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America**, v. 50, n. 11, p. 1477-1486, 2010.

LEINONEN, M.; SÄKKINEN, A.; KALLIOKOSKI, R.; LUOTONEN, J.; TIMONEN, M.; MÄKELÄ, P. H. Antibody response to 14-valent pneumococcal capsular polysaccharide vaccine in pre-school age children. **Pediatr. Infect. Dis.**, v. 5, p. 39-44, 1986.

LEMESSURIER KS, HACKER H, CHI L, TUOMANEN E, REDECKE V. Type I Interferon Protects against Pneumococcal Invasive Disease by Inhibiting Bacterial Transmigration across the Lung. **PLoS Pathog.** v. 9, n. 11, p. e1003727, 2013

LIMA, F. A.; FERREIRA, D. M.; MORENO, A. T.; FERREIRA, P. C.; PALMA, G. M.; FERREIRA, J. M., JR.; RAW, I.; MIYAJI, E. N.; HO, P. L.; OLIVEIRA, M. L. Controlled inflammatory responses in the lungs are associated with protection elicited by a pneumococcal

surface protein A-based vaccine against a lethal respiratory challenge with *Streptococcus pneumoniae* in mice. **Clin. Vaccine Immunol.**, v. 19, n. 9, p. 1382-1392, 2012.

LIU; JOHNSON, H. L.; COUSENS, S.; PERIN, J.; SCOTT, S.; LAWN, J. E.; RUDAN, I.; CAMPBELL, H.; CIBULSKIS, R.; LI, M.; MATHERS, C.; BLACK, R. E. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. **Lancet**, v. 379, n. 9832, p. 2151-2161, 2012.

LIU, S.; TOBIAS, R.; MCCLURE, S.; STYBA, G.; SHI, Q.; JACKOWSKI, G. Removal of endotoxin from recombinant protein preparations. **Clinical Biochemistry**, v. 30, n. 6, p. 455-463, 1997.

LU, Y. J.; GROSS, J.; BOGAERT, D.; FINN, A.; BAGRADE, L.; ZHANG, Q.; KOLLS, J. K.; SRIVASTAVA, A.; LUNDGREN, A.; FORTE, S.; THOMPSON, C. M.; HARNEY, K. F.; ANDERSON, P. W.; LIPSITCH, M.; MALLEY, R. Interleukin-17A mediates acquired immunity to pneumococcal colonization. **PLoS Pathogens**, v. 4, n. 9, p. e1000159, 2008.

LU, Y. J.; FORTE, S.; THOMPSON, C. M.; ANDERSON, P. W.; MALLEY, R. Protection against Pneumococcal colonization and fatal pneumonia by a trivalent conjugate of a fusion protein with the cell wall polysaccharide. **Infect. Immun.**, v. 77, n. 5, p. 2076-2083, 2009.

MALLEY, R.; LIPSITCH, M.; STACK, A.; SALADINO, R.; FLEISHER, G.; PELTON, S.; THOMPSON, C.; BRILES, D.; ANDERSON, P. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. **Infect. Immun.**, v. 69, n. 8, p. 4870-4873, 2001.

MALLEY, R.; HENNEKE, P.; MORSE, S. C.; CIESLEWICZ, M. J.; LIPSITCH, M.; THOMPSON, C. M.; KURT-JONES, E.; PATON, J. C.; WESSELS, M. R.; GOLENBOCK, D. T. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. **Proc. Natl.A. Sci. U.S.A.**, v. 100, n. 4, p. 1966-1971, 2003.

MALLEY, R.; TRZCINSKI, K.; SRIVASTAVA, A.; THOMPSON, C. M.; ANDERSON, P. W.; LIPSITCH, M. CD4⁺ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. **Proc. Natl. Acad. Sci. U.S.A.**, v. 102, n. 13, p. 4848-4853, 2005.

MALLEY, R.; SRIVASTAVA, A.; LIPSITCH, M.; THOMPSON, C. M.; WATKINS, C.; TZIANABOS, A.; ANDERSON, P. W. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. **Infect. Immun.**, v. 74, n. 4, p. 2187-2195, 2006.

MARTNER, A.; SKOVBJERG, S.; PATON, J. C.; WOLD, A. E. *Streptococcus pneumoniae* autolysis prevents phagocytosis and production of phagocyte-activating cytokines. **Infect. Immun.**, v. 77, n. 9, p. 3826-3837, 2009.

MCDANIEL, L. S.; SCOTT, G.; WIDENHOFER, K.; CARROLL, J. M.; BRILES, D. E. Analysis of a surface protein of *Streptococcus pneumoniae* recognised by protective monoclonal antibodies. **Microb. Pathog.**, v. 1, n. 6, p. 519-531, 1986.

MCDANIEL, L. S.; RALPH, B. A.; MCDANIEL, D. O.; BRILES, D. E. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. **Microb. Pathog.**, v. 17, n. 5, p. 323-337, 1994.

MCNEELA, E. A.; BURKE, A.; NEILL, D. R.; BAXTER, C.; FERNANDES, V. E.; FERREIRA, D.; SMEATON, S.; EL-RACHKIDY, R.; MCLOUGHLIN, R. M.; MORI, A.; MORAN, B.; FITZGERALD, K. A.; TSCHOPP, J.; PETRILLI, V.; ANDREW, P. W.; KADIOGLU, A.; LAVELLE, E. C. Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. **PLoS Pathogens**, v. 6, n. 11, p. e1001191, 2010.

MEHR, S.; WOOD, N. *Streptococcus pneumoniae* - a review of carriage, infection, serotype replacement and vaccination. **Paediatric Respiratory Reviews**, v. 13, n. 4, p. 258-264, 2012.

MELIN, M.; JARVA, H.; SIIRA, L.; MERI, S.; KAYHTY, H.; VAKEVAINEN, M. *Streptococcus pneumoniae* capsular serotype 19F is more resistant to C3 deposition and less sensitive to opsonophagocytosis than serotype 6B. **Infect. Immun.**, v. 77, n. 2, p. 676-684, 2009.

MITCHELL, T. J.; ANDREW, P. W.; SAUNDERS, F. K.; SMITH, A. N.; BOULNOIS, G. J. Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. **Mol. Microbiol.**, v. 5, n. 8, p. 1883-1888, 1991.

MOFFITT, K.; SKOBERNE, M.; HOWARD, A.; GAVRILESCU, L. C.; GIERAHN, T.; MUNZER, S.; DIXIT, B.; GIANNASCA, P.; FLECHTNER, J. B.; MALLEY, R. Toll-like receptor 2-dependent protection against pneumococcal carriage by immunization with lipidated pneumococcal proteins. **Infect. Immun.**, v. 82, n. 5, p. 2079-2086, 2014.

MOFFITT, K. L.; GIERAHN, T. M.; LU, Y. J.; GOUVEIA, P.; ALDERSON, M.; FLECHTNER, J. B.; HIGGINS, D. E.; MALLEY, R. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. **Cell Host & Microbe**, v. 9, n. 2, p. 158-165, 2011.

MOHAN, T.; VERMA, P.; RAO, D. N. Novel adjuvants & delivery vehicles for vaccines development: a road ahead. **Indian J. Med. Res.**, v. 138, n. 5, p. 779-795, 2013.

MOLLERACH, M.; REGUEIRA, M.; BONOFIOLIO, L.; CALLEJO, R.; PACE, J.; DI FABIO, J. L.; HOLLINGSHEAD, S.; BRILES, D. Invasive *Streptococcus pneumoniae* isolates from Argentinian children: serotypes, families of pneumococcal surface protein A (PspA) and genetic diversity. **Epidemiology and Infection**, v. 132, n. 2, p. 177-184, 2004.

MOOK-KANAMORI, B. B.; GELDHOFF, M.; VAN DER POLL, T.; VAN DE BEEK, D. Pathogenesis and pathophysiology of pneumococcal meningitis. **Clin. Microbiol. Rev.**, v. 24, n. 3, p. 557-591, 2011.

MORENO, A. T.; OLIVEIRA, M. L.; FERREIRA, D. M.; HO, P. L.; DARRIEUX, M.; LEITE, L. C.; FERREIRA, J. M., JR.; PIMENTA, F. C.; ANDRADE, A. L.; MIYAJI, E. N. Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. **Clin. Vaccine Immunol.**, v. 17, n. 3, p. 439-446, 2011.

NABORS, G. S.; BRAUN, P. A.; HERRMANN, D. J.; HEISE, M. L.; PYLE, D. J.; GRAVENSTEIN, S.; SCHILLING, M.; FERGUSON, L. M.; HOLLINGSHEAD, S. K.; BRILES, D. E.; BECKER, R. S. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. **Vaccine**, v. 18, n. 17, p. 1743-1754, 2000.

NASCIMENTO, I. P.; DIAS, W. O.; MAZZANTINI, R. P.; MIYAJI, E. N.; GAMBERINI, M.; QUINTILIO, W.; GEBARA, V. C.; CARDOSO, D. F.; HO, P. L.; RAW, I.; WINTER, N.; GICQUEL, B.; RAPPUOLI, R.; LEITE, L. C. Recombinant Mycobacterium bovis BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live Bordetella pertussis in mice. **Infect. Immun.**, v. 68, n. 9, p. 4877-4883, 2000.

NASCIMENTO, I. P.; DIAS, W. O.; QUINTILIO, W.; CHRIST, A. P.; MORAES, J. F.; VANCETTO, M. D.; RIBEIRO-DOS-SANTOS, G.; RAW, I.; LEITE, L. C. Neonatal immunization with a single dose of recombinant BCG expressing subunit S1 from pertussis toxin induces complete protection against Bordetella pertussis intracerebral challenge. **Microbes Infect.**, v. 10, n. 2, p. 198-202, 2008.

NGUYEN, C. T.; KIM, S. Y.; KIM, M. S.; LEE, S. E.; RHEE, J. H. Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against Streptococcus pneumoniae infection in mice. **Vaccine**, v. 29, n. 34, p. 5731-5739, 2011.

O'BRIEN, K. L.; STEINHOFF, M. C.; EDWARDS, K.; KEYSERLING, H.; THOMS, M. L.; MADORE, D. Immunologic priming of young children by pneumococcal glycoprotein conjugate, but not polysaccharide, vaccines. **Pediatr. Infect. Dis. J.**, v. 15, n., p. 425-430, 1996.

O'BRIEN, K. L.; WOLFSON, L. J.; WATT, J. P.; HENKLE, E.; DELORIA-KNOLL, M.; MCCALL, N.; LEE, E.; MULHOLLAND, K.; LEVINE, O. S.; CHERIAN, T. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. **Lancet**, v. 374, n. 9693, p. 893-902, 2009.

O'HAGAN, D. T.; RAPPUOLI, R.; DE GREGORIO, E.; TSAI, T.; DEL GIUDICE, G. MF59 adjuvant: the best insurance against influenza strain diversity. **Expert Rev. Vaccines**, v. 10, n. 4, p. 447-462, 2011.

OGUNNIYI, A. D.; FOLLAND, R. L.; BRILES, D. B.; HOLLINGSHEAD, S. K.; PATON, J. C. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. **Infect. Immun.**, v. 68, n. 5, p. 3028-3033, 2000.

PAATS, M. S.; BERGEN, I. M.; HANSELAAR, W. E.; GROENINX VAN ZOELLEN, E. C.; HOOGSTEDEN, H. C.; HENDRIKS, R. W.; VAN DER EERDEN, M. M. Local and systemic cytokine profiles in nonsevere and severe community-acquired pneumonia. **The European Respiratory Journal**, v. 41, n. 6, p. 1378-1385, 2013.

PANCHOLI, V.; FONTAN, P.; JIN, H. Plasminogen-mediated group A streptococcal adherence to and pericellular invasion of human pharyngeal cells. **Microb. Pathog.**, v. 35, n. 6, p. 293-303, 2003.

PATON, J. C.; FERRANTE, A. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. **Infect. Immun.**, v. 41, n. 3, p. 1212-1216, 1983.

PATON, J. C.; LOCK, R. A.; HANSMAN, D. J. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. **Infect. Immun.**, v. 40, n. 2, p. 548-552, 1983.

PATON, J. C.; ROWAN-KELLY, B.; FERRANTE, A. Activation of human complement by the pneumococcal toxin pneumolysin. **Infect. Immun.**, v. 43, n. 3, p. 1085-1087, 1984.

PATON, J. C.; LOCK, R. A.; LEE, C. J.; LI, J. P.; BERRY, A. M.; MITCHELL, T. J.; ANDREW, P. W.; HANSMAN, D.; BOULNOIS, G. J. Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. **Infect. Immun.**, v. 59, n. 7, p. 2297-2304, 1991.

PATON, J. C. Vaccines against *Streptococcus pneumoniae*. In: BAGNOLI, R. R. A. F. (Ed.). **Vaccine Design - innovative approaches and novel strategies**. Caister Academic Press, 2011. p. 303-321.

PERCIANI, C. T.; BARAZZONE, G. C.; GOULART, C.; CARVALHO, E.; CABRERA-CRESPO, J.; GONCALVES, V. M.; LEITE, L. C.; TANIZAKI, M. M. Conjugation of polysaccharide 6B from *Streptococcus pneumoniae* with pneumococcal surface protein A: PspA conformation and its effect on the immune response. **Clin. Vaccine Immunol.**, v. 20, n. 6, p. 858-866, 2013.

PIMENTA, F. C.; RIBEIRO-DIAS, F.; BRANDILEONE, M. C.; MIYAJI, E. N.; LEITE, L. C.; SGAMBATTI DE ANDRADE, A. L. Genetic diversity of PspA types among nasopharyngeal isolates collected during an ongoing surveillance study of children in Brazil. **J. Clin. Microbiol.**, v. 44, n. 8, p. 2838-2843, 2006.

POLAND, G. A. The burden of pneumococcal disease: the role of conjugate vaccines. **Vaccine**, v. 17, n. 13-14, p. 1674-1679, 1999.

PRICE, K. E.; CAMILLI, A. Pneumolysin localizes to the cell wall of *Streptococcus pneumoniae*. **J. Bacteriol.**, v. 191, n. 7, p. 2163-2168, 2009.

PRICE, K. E.; GREENE, N. G.; CAMILLI, A. Export requirements of pneumolysin in *Streptococcus pneumoniae*. **J. Bacteriol.**, v. 194, n. 14, p. 3651-3660, 2012.

PRYMULA, R.; HANOVCOVA, I.; SPLINO, M.; KRIZ, P.; MOTLOVA, J.; LEBEDOVA, V.; LOMMEL, P.; KALISKOVA, E.; PASCAL, T.; BORYS, D.; SCHUERMAN, L. Impact of the 10-valent pneumococcal non-typeable *Haemophilus influenzae* Protein D conjugate vaccine (PHiD-CV) on bacterial nasopharyngeal carriage. **Vaccine**, v. 29, n. 10, p. 1959-1967, 2011.

PRYMULA, R.; PAZDIORA, P.; TRASKINE, M.; RUGGEBERG, J. U.; BORYS, D. Safety and immunogenicity of an investigational vaccine containing two common pneumococcal proteins in toddlers: a phase II randomized clinical trial. **Vaccine**, v. 32, n. 25, p. 3025-3034, 2014.

RAMOS, C. R.; ABREU, P. A.; NASCIMENTO, A. L.; HO, P. L. A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. **Braz. J. Med. Biol. Res.**, v. 37, n. 8, p. 1103-1109, 2004.

REN, B.; SZALAI, A. J.; THOMAS, O.; HOLLINGSHEAD, S. K.; BRILES, D. E. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of Streptococcus pneumoniae. **Infect. Immun.**, v. 71, n. 1, p. 75-85, 2003.

REN, B.; SZALAI, A. J.; HOLLINGSHEAD, S. K.; BRILES, D. E. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. **Infect. Immun.**, v. 72, n. 1, p. 114-122, 2004.

REYRAT, J. M.; BERTHET, F. X.; GICQUEL, B. The urease locus of Mycobacterium tuberculosis and its utilization for the demonstration of allelic exchange in Mycobacterium bovis bacillus Calmette-Guerin. **Proc. Natl. Acad. Sci. U.S.A.**, v. 92, n. 19, p. 8768-8772, 1995.

RICHTER, S. S.; DIEKEMA, D. J.; HEILMANN, K. P.; DOHRN, C. L.; RIAHI, F.; DOERN, G. V. Changes in pneumococcal serotypes and antimicrobial resistance after introduction of the 13-valent conjugate vaccine in the United States. **Antimicrobial Agents and Chemotherapy**, v. 58, n. 11, p. 6484-6489, 2014.

ROCHE, H.; HAKANSSON, A.; HOLLINGSHEAD, S. K.; BRILES, D. E. Regions of PspA/EF3296 best able to elicit protection against Streptococcus pneumoniae in a murine infection model. **Infect. Immun.**, v. 71, n. 3, p. 1033-1041, 2003.

RODRIGUES, D. C., B.S.; ABREU-DE-OLIVEIRA, J.T.; MOREIRA, R. A. AND RUSSO, M. Differences in Macrophages stimulation and leukocyte Accumulation in response to Intraperitoneal administration of Glucose/Mannose-Binding Plant Lectins. **Brazilian J. Med. Biol. Res.**, v. 25, n., p. 823-826, 1992.

RODRIGUES, M. R.; RODRIGUEZ, D.; RUSSO, M.; CAMPA, A. Macrophage activation includes high intracellular myeloperoxidase activity. **Biochemical and Biophysical Research Communications**, v. 292, n. 4, p. 869-873, 2002.

ROSSJOHN, J.; GILBERT, R. J.; CRANE, D.; MORGAN, P. J.; MITCHELL, T. J.; ROWE, A. J.; ANDREW, P. W.; PATON, J. C.; TWETEN, R. K.; PARKER, M. W. The molecular mechanism of pneumolysin, a virulence factor from Streptococcus pneumoniae. **J. Mol. Biol.**, v. 284, n. 2, p. 449-461, 1998.

RUBINS, J. B.; DUANE, P. G.; CHARBONEAU, D.; JANOFF, E. N. Toxicity of pneumolysin to pulmonary endothelial cells in vitro. **Infect. Immun.**, v. 60, n. 5, p. 1740-1746, 1992.

SABLE, S. B.; CHERUVU, M.; NANDAKUMAR, S.; SHARMA, S.; BANDYOPADHYAY, K.; KELLAR, K. L.; POSEY, J. E.; PLIKAYTIS, B. B.; AMARA, R. R.; SHINNICK, T. M. Cellular immune responses to nine Mycobacterium tuberculosis vaccine candidates following intranasal vaccination. **PLoS One**, v. 6, n. 7, p. e22718, 2011.

SALHA, D.; SZETO, J.; MYERS, L.; CLAUS, C.; SHEUNG, A.; TANG, M.; LJUTIC, B.; HANWELL, D.; OGILVIE, K.; MING, M.; MESSHAM, B.; VAN DEN DOBBELSTEEN, G.; HOPFER, R.; OCHS, M. M.; GALLICHAN, S. Neutralizing antibodies elicited by a novel detoxified pneumolysin derivative, PlyD1, provide protection against both pneumococcal infection and lung injury. **Infect. Immun.**, v. 80, n. 6, p. 2212-2220, 2012.

SCOTTA, M. C.; VERAS, T. N.; KLEIN, P. C.; TRONCO, V.; POLACK, F. P.; MATTIELLO, R.; PITREZ, P. M.; JONES, M. H.; STEIN, R. T.; PINTO, L. A. Impact of 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) on childhood pneumonia hospitalizations in Brazil two years after introduction. **Vaccine**, v. 32, n. 35, p. 4495-4499, 2014.

SEIBERLING, M.; BOLOGA, M.; BROOKES, R.; OCHS, M.; GO, K.; NEVEU, D.; KAMTCHOUA, T.; LASHLEY, P.; YUAN, T.; GURUNATHAN, S. Safety and immunogenicity of a pneumococcal histidine triad protein D vaccine candidate in adults. **Vaccine**, v. 30, n. 52, p. 7455-7460, 2012.

SHAPER, M.; HOLLINGSHEAD, S. K.; BENJAMIN, W. H., JR.; BRILES, D. E. PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. **Infect. Immun.**, v. 72, n. 9, p. 5031-5040, 2004.

SHRIVE, A. K.; CHEETHAM, G. M.; HOLDEN, D.; MYLES, D. A.; TURNELL, W. G.; VOLANAKIS, J. E.; PEPYS, M. B.; BLOOMER, A. C.; GREENHOUGH, T. J. Three dimensional structure of human C-reactive protein. **Nature Structural Biology**, v. 3, n. 4, p. 346-354, 1996.

SINGH, R.; SINGH, S.; SHARMA, P. K.; SINGH, U. P.; BRILES, D. E.; HOLLINGSHEAD, S. K.; LILLARD, J. W., JR. Helper T cell epitope-mapping reveals MHC-peptide binding affinities that correlate with T helper cell responses to pneumococcal surface protein A. **PLoS One**, v. 5, n. 2, p. e9432, 2010.

SIVAKUMAR, S. M.; SAFHI, M. M.; KANNADASAN, M.; SUKUMARAN, N. Vaccine adjuvants - Current status and prospects on controlled release adjuvancity. **Saudi Pharmaceutical Journal:SPJ: the Official Publication of the Saudi Pharmaceutical Society**, v. 19, n. 4, p. 197-206, 2011.

SIXSMITH, J. D.; PANAS, M. W.; LEE, S.; GILLARD, G. O.; WHITE, K.; LIFTON, M. A.; BALACHANDRAN, H.; MACH, L.; MILLER, J. P.; LAVINE, C.; DEMARCO, C. T.; TOMARAS, G. D.; GEE, C.; PORCELLI, S. A.; LARSEN, M. H.; FROTHINGHAM, R.; SCHMITZ, J. E.; JACOBS, W. R., JR.; HAYNES, B. F.; LETVIN, N. L.; KORIOTH-SCHMITZ, B. Recombinant Mycobacterium bovis bacillus Calmette-Guerin vectors prime for strong cellular responses to simian immunodeficiency virus gag in rhesus macaques. **Clin. Vaccine Immunol.**, v. 21, n. 10, p. 1385-1395, 2014.

SNAPPER, C. M.; ROSAS, F. R.; JIN, L.; WORTHAM, C.; KEHRY, M. R.; MOND, J. J. Bacterial lipoproteins may substitute for cytokines in the humoral immune response to T cell-independent type II antigens. **J. Immunol.**, v. 155, n. 12, p. 5582-5589, 1995.

SPELLERBERG, B.; CUNDELL, D. R.; SANDROS, J.; PEARCE, B. J.; IDANPAAN-HEIKKILA, I.; ROSENOW, C.; MASURE, H. R. Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. **Mol. Microbiol.**, v. 19, n. 4, p. 803-813, 1996.

STEINER, S. L., D.; KEYSERLING, H.L.; CARLONE, G.M. . *Streptococcus pneumoniae* opsonophagocytosis using differentiated HL-60 cells **Center for Disease Control and Prevention and Emory University Atlanta, GA**, 1999.

SUN, R.; SKEIKY, Y. A.; IZZO, A.; DHEENADHAYALAN, V.; IMAM, Z.; PENN, E.; STAGLIANO, K.; HADDOCK, S.; MUELLER, S.; FULKERSON, J.; SCANGA, C.; GROVER, A.; DERRICK, S. C.; MORRIS, S.; HONE, D. M.; HORWITZ, M. A.; KAUFMANN, S. H.; SADOFF, J. C. Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with *Mycobacterium tuberculosis*. **Vaccine**, v. 27, n. 33, p. 4412-4423, 2009.

SUPPLY, P.; SUTTON, P.; COUGHLAN, S. N.; BILO, K.; SAMAN, E.; TREES, A. J.; CESBRON DELAUW, M. F.; LOCHT, C. Immunogenicity of recombinant BCG producing the GRA1 antigen from *Toxoplasma gondii*. **Vaccine**, v. 17, n. 7-8, p. 705-714, 1999.

SWIATLO, E.; KING, J.; NABORS, G. S.; MATHEWS, B.; BRILES, D. E. Pneumococcal surface protein A is expressed in vivo, and antibodies to PspA are effective for therapy in a murine model of pneumococcal sepsis. **Infect. Immun.**, v. 71, n. 12, p. 7149-7153, 2003.

TRZCINSKI, K.; THOMPSON, C.; MALLEY, R.; LIPSITCH, M. Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. **Infect. Immun.**, v. 73, n. 10, p. 7043-7046, 2005.

TSENG, H. J.; MCEWAN, A. G.; PATON, J. C.; JENNINGS, M. P. Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. **Infect. Immun.**, v. 70, n. 3, p. 1635-1639, 2002.

TU, A. H.; FULGHAM, R. L.; MCCRORY, M. A.; BRILES, D. E.; SZALAI, A. J. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. **Infect. Immun.**, v. 67, n. 9, p. 4720-4724, 1999.

VADESILHO, C. F.; FERREIRA, D. M.; GORDON, S. B.; BRILES, D. E.; MORENO, A. T.; OLIVEIRA, M. L.; HO, P. L.; MIYAJI, E. N. Mapping of epitopes recognized by antibodies induced by immunization of mice with PspA and PspC. **Clin. Vaccine Immunol.**, v. 21, n. 7, p. 940-948, 2014.

VAN GINKEL, F. W.; MCGHEE, J. R.; WATT, J. M.; CAMPOS-TORRES, A.; PARISH, L. A.; BRILES, D. E. Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. **Proc. Natl. Acad. Sci. U. S. A.**, v. 100, n. 24, p. 14363-14367, 2003.

VARALDO, P. B.; LEITE, L. C.; DIAS, W. O.; MIYAJI, E. N.; TORRES, F. I.; GEBARA, V. C.; ARMOA, G. R.; CAMPOS, A. S.; MATOS, D. C.; WINTER, N.; GICQUEL, B.; VILAR, M. M.; MCFADDEN, J.; ALMEIDA, M. S.; TENDLER, M.; MCINTOSH, D. Recombinant

Mycobacterium bovis BCG expressing the Sm14 antigen of *Schistosoma mansoni* protects mice from cercarial challenge. **Infect. Immun.**, v. 72, n. 6, p. 3336-3343, 2004.

VASCONCELLOS, H. L.; SCARAMUZZI, K.; NASCIMENTO, I. P.; DA COSTA FERREIRA, J. M., JR.; ABE, C. M.; PIAZZA, R. M.; KIPNIS, A.; DIAS DA SILVA, W. Generation of recombinant bacillus Calmette-Guerin and *Mycobacterium smegmatis* expressing BfpA and intimin as vaccine vectors against enteropathogenic *Escherichia coli*. **Vaccine**, v. 30, n. 41, p. 5999-6005, 2012.

VELA CORAL, M. C.; FONSECA, N.; CASTANEDA, E.; DI FABIO, J. L.; HOLLINGSHEAD, S. K.; BRILES, D. E. Pneumococcal surface protein A of invasive *Streptococcus pneumoniae* isolates from Colombian children. **Emerging Infectious Diseases**, v. 7, n. 5, p. 832-836, 2001.

VESIKARI, T.; WYSOCKI, J.; CHEVALLIER, B.; KARVONEN, A.; CZAJKA, H.; ARSENE, J. P.; LOMMEL, P.; DIEUSSAERT, I.; SCHUERMAN, L. Immunogenicity of the 10-valent pneumococcal non-typeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine. **The Pediatric Infectious Disease Journal**, v. 28, n. 4, p. S66-76, 2009. Suppl.

VOLLMER, W.; TOMASZ, A. Peptidoglycan N-acetylglucosamine deacetylase, a putative virulence factor in *Streptococcus pneumoniae*. **Infect. Immun.**, v. 70, n. 12, p. 7176-7178, 2002.

WADWA, R. P.; FEIGIN, R. D. Pneumococcal vaccine: an update. **Pediatrics**, v. 103, n. 5, pt 1, p. 1035-1037, 1999.

WEINBERGER, D. M.; MALLEY, R.; LIPSITCH, M. Serotype replacement in disease after pneumococcal vaccination. **Lancet**, v. 378, n. 9807, p. 1962-1973, 2011.

WEISER, J. N.; BAE, D.; FASCHING, C.; SCAMURRA, R. W.; RATNER, A. J.; JANOFF, E. N. Antibody-enhanced pneumococcal adherence requires IgA1 protease. **Proc. Natl. Acad. Sci. U.S.A.**, v. 100, n. 7, p. 4215-4220, 2003.

WINKELSTEIN, J. A.; ABRAMOVITZ, A. S.; TOMASZ, A. Activation of C3 via the alternative complement pathway results in fixation of C3b to the pneumococcal cell wall. **J. Immunol.**, v. 124, n. 5, p. 2502-2506, 1980.

WIZEMANN, T. M.; MOSKOVITZ, J.; PEARCE, B. J.; CUNDELL, D.; ARVIDSON, C. G.; SO, M.; WEISSBACH, H.; BROTH, N.; MASURE, H. R. Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens. **Proc. Natl. Acad. Sci. U. S. A.**, v. 93, n. 15, p. 7985-7990, 1996.

WORLD HEALTH ORGANIZATION. PNEUMOCOCCAL VACCINES. **Weekly Epidemiological Record**, v. 78, n., p. 97-120, 2003.

WORLD HEALTH ORGANIZATION, 2009. **Immunization Surveillance, Assessment and Monitoring**. Disponível em:

<http://www.who.int/immunization_monitoring/burden/Pneumo_incidence_map.JPG>. Acesso: 20 fev. 2013.

WORLD HEALTH ORGANIZATION. Pneumococcal vaccines WHO position paper - 2012 - recommendations. **Vaccine**, v. 30, n. 32, p. 4717-4718, 2012.

WORLD HEALTH ORGANIZATION. “**Global Immunization Data – July 2014**”; WHO/ UNICEF. Disponível em: <http://www.who.int/hpvcentre/Global_Immunization_Data.pdf>. Acesso: 14 set. 2014.

YOTHER, J. Capsules. In: TUOMANEN, E. I., MITCHELL, T. J. DONALD A. M.; BRIAN G. SPRATT (Ed.). **The pneumococcus**. Washington, D.C.: ASM Press, 2004, p.30-48.

YUSTE, J.; BOTTO, M.; PATON, J. C.; HOLDEN, D. W.; BROWN, J. S. Additive inhibition of complement deposition by pneumolysin and PspA facilitates *Streptococcus pneumoniae* septicemia. **J. Immunol.**, v. 175, n. 3, p. 1813-1819, 2005.

ZHANG, J. R.; MOSTOV, K. E.; LAMM, M. E.; NANNO, M.; SHIMIDA, S.; OHWAKI, M.; TUOMANEN, E. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. **Cell**, v. 102, n. 6, p. 827-837, 2000.

APÊNDICE A – Alinhamento das sequências de aminoácidos das Pspa utilizadas

[illegible]

Sequência selecionada em vermelho caixa vermelha corresponde à região definidora de clados (CDR)

APÊNDICE B – Artigos publicados

I. GOULART, CIBELLY; SILVA, THAIS RAQUEL DA; RODRIGUEZ, DUNIA; POLITANO, WALTER RODRIGO; LEITE, LUCIANA C. C.; DARRIEUX, MICHELLE. Characterization of Protective Immune Responses Induced by Pneumococcal Surface Protein A in Fusion with Pneumolysin Derivatives. **Plos One**, v. 8, p. e59605, 2013.

II. DARRIEUX, MICHELLE; GOULART, CIBELLY; BRILES, DAVID; LEITE, LUCIANA CEZAR DE CERQUEIRA. Current status and perspectives on protein-based pneumococcal vaccines. **Critical Reviews in Microbiology**, v. 1, p. 1-11, 2013.

III. DE AMICIS, KARINE M.; DE BARROS, SAMAR FRESCHI; ALENCAR, RAQUEL E.; POSTÓL, EDILBERTO; MARTINS, CARLO DE OLIVEIRA; ARCURI, HELEN ANDRADE; GOULART, CIBELLY; KALIL, JORGE; GUILHERME, LUIZA. Analysis of the coverage capacity of the StreptInCor candidate vaccine against *Streptococcus pyogenes*. **Vaccine** (Guildford), v. 13, p. 1134-1, 2013.

IV. PERCIANI, C. T.; BARAZZONE, G. C.; GOULART, C.; CARVALHO, E.; CABRERA-CRESPO, J.; GONCALVES, V. M.; LEITE, L. C. C.; TANIZAKI, M. M. Conjugation of Polysaccharide 6B from *Streptococcus pneumoniae* with Pneumococcal Surface Protein A: PspA conformation and its effect on the immune response. **Clinical and Vaccine Immunology**, v. prelo, p. online, 2013.

V. SANTAMARIA, RAQUEL; GOULART, C.; PERCIANI, CATIA T.; BARAZZONE, GIOVANA C.; CARVALHO, RIMENYS JR.; GONÇALVES, VIVIANE M.; LEITE, LUCIANA C.C.; TANIZAKI, MARTHA M. Humoral immune response of a pneumococcal conjugate vaccine: Capsular polysaccharide serotype 14 Lysine modified PspA. **Vaccine** (Guildford), v. 29, p. 8689-8695, 2011.

VI. GOULART, CIBELLY; DARRIEUX, MICHELLE; RODRIGUEZ, DUNIA; PIMENTA, FABIANA C.; BRANDILEONE, MARIA CRISTINA C.; DE ANDRADE, ANA LUCIA S.S.; LEITE, LUCIANA C.C. Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. **Vaccine** (Guildford), v. 29, p. 1634-1642, 2011.

VII. BARAZZONE, GIOVANA C.; CARVALHO, RIMENYS; KRASCHOWETZ, STEFANIE; HORTA, ANTONIO L.; SARGO, CÍNTIA R.; SILVA, ADILSON J.; ZANGIROLAMI, TERESA C.; GOULART, CIBELLY; LEITE, LUCIANA C.C.; TANIZAKI, MARTHA M.; GONÇALVES, VIVIANE M.; CABRERA-CRESPO, JOAQUIN. Production and purification of recombinant fragment of pneumococcal surface protein A (PspA) in *Escherichia coli*. **Procedia Vaccinology**, v. 4, p. 27-35, 2011.

Characterization of Protective Immune Responses Induced by Pneumococcal Surface Protein A in Fusion with Pneumolysin Derivatives

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Abstract

Pneumococcal surface protein A (PspA) and Pneumolysin derivatives (Pds) are important vaccine candidates, which can confer protection in different models of pneumococcal infection. Furthermore, the combination of these two proteins was able to increase protection against pneumococcal sepsis in mice. The present study investigated the potential of hybrid proteins generated by genetic fusion of PspA fragments to Pds to increase cross-protection against fatal pneumococcal infection. Pneumolysoids were fused to the N-terminus of clade 1 or clade 2 *pspA* gene fragments. Mouse immunization with the fusion proteins induced high levels of antibodies against PspA and Pds, able to bind to intact pneumococci expressing a homologous PspA with the same intensity as antibodies to rPspA alone or the co-administered proteins. However, when antibody binding to pneumococci with heterologous PspAs was examined, antisera to the PspA-Pds fusion molecules showed stronger antibody binding and C3 deposition than antisera to co-administered proteins. In agreement with these results, antisera against the hybrid proteins were more effective in promoting the phagocytosis of bacteria bearing heterologous PspAs *in vitro*, leading to a significant reduction in the number of bacteria when compared to co-administered proteins. The respective antisera were also capable of neutralizing the lytic activity of Pneumolysin on sheep red blood cells. Finally, mice immunized with fusion proteins were protected against fatal challenge with pneumococcal strains expressing heterologous PspAs. Taken together, the results suggest that PspA-Pd fusion proteins comprise a promising vaccine strategy, able to increase the immune response mediated by cross-reactive antibodies and complement deposition to heterologous strains, and to confer protection against fatal challenge.

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Introduction

Streptococcus pneumoniae is a major human pathogen, accounting for over 10% of total deaths in children under the age of five [1]. Despite the well established efficacy of conjugate vaccines against invasive disease, the high production costs involved in the conjugation processes limit their implementation in lower income countries, in which the burden of pneumococcal diseases is highest. Also, due to the limited number of polysaccharides included in the formulations, the extent of vaccine coverage tends to decrease as less prevalent serotypes emerge. In fact, serotype replacement has been observed after the introduction of PCV7 in different populations [2,3]. Finally, serotype replacement is associated with the emergence of antibiotic resistant clones [4], reinforcing the need for cost-effective strategies that confer broad protection, such as protein-based vaccines.

PspA and Pneumolysin (Ply) are among the most well studied pneumococcal proteins; their contribution to virulence has been demonstrated with mutant strains lacking either one or both proteins, which have shown reduced fitness in different models of

colonization, lung infection and bacteremia [5]. Mutant strains were cleared more rapidly from the lungs and blood of mice when compared to wild type counterparts [5,6] and deposited more C3 *in vitro* [7]. Furthermore, the combination of both mutations had an additive effect on C3 deposition and pneumococcal clearance [6], suggesting that these proteins contribute synergistically to bacterial evasion of innate immune responses [6,7].

Recombinant forms of PspA and Pneumolysin derivatives (Pds) have been investigated as potential vaccine candidates in different animal models, with promising results. The N-terminal region of PspA, which is responsible for inhibiting complement deposition on the bacterial surface [8,9] and contains most of the immunogenic epitopes of the molecule [10], confers protection against invasive infection [11–13], lobar pneumonia [14] and colonization [15,16]. Furthermore, it has been recently demonstrated that maternal immunization with PspA protects the offspring against pneumococcal infection [17]. The N-terminus of PspA, however, exhibits structural and serological variability [18]. Based on the observation that different PspA molecules induce antibodies with distinct degrees of cross-reactivity [19,20]

and cross-protection [11,21], it has been suggested that PspA-based anti-pneumococcal vaccines should include more than one molecule in order to extend coverage. The potential of PspA as a vaccine candidate has been further supported by human clinical trials, which have demonstrated the induction of antibodies with high cross-reactivity against heterologous molecules [21], which can passively protect mice against fatal pneumococcal infection [21].

Pneumolysin (Ply) is a cholesterol dependent cytolysin with several biological effects, such as activation of classical complement pathway [22], induction of apoptosis in numerous cells types [23,24], impairment of ciliary function in the lungs and induction of oxidative burst by neutrophils [22]. In fact, the instillation of purified Ply in the lungs is sufficient to reproduce many aspects of pneumococcal pneumonia in rats (reviewed in [22]). Furthermore, Ply has been shown to interact with TLR-4 [25] and to induce TLR-4 independent activation of the NLRP3 inflammasome, contributing to host protection against pneumococcal pneumonia [26] and lethal infection [25].

Since Ply is toxic in its native form, several detoxified forms – named pneumolysoids (Pds) – have been produced, by site-directed mutagenesis or chemical detoxification, and evaluated for their immunogenicity and protective effect in different animal models, with variable results, including protection in rhesus macaques [14,27–33]. Of those toxoids, the best characterized are PdB, carrying a Trp-Phe substitution at position 433 [30], and PdT, a triple mutant containing Asp-385 to Asn, Cys-428 to Gly and Trp-433 to Phe substitutions [34]. While PdT alone or co-administered with other pneumococcal antigens, did not induce significant protection against lethal intraperitoneal challenge [29], PdB has been shown to elicit protection against nasal challenge with some pneumococcal strains, which was enhanced by co-administration of other pneumococcal proteins, such as PspA and PhTB [14,35]. The combination of PspA and PdB elicited the highest protection levels in mouse models of sepsis and focal pneumonia, suggesting a complementary role for these two antigens [14,36].

On a whole, the results indicate that effective protein-based anti-pneumococcal vaccines tend to require the combination of different proteins in order to extend protection. In the present work, we investigated the ability of fusion proteins including the N-terminal region of family 1 PspAs and detoxified derivatives of Pneumolysin to induce protective immune responses in a mouse model of fatal pneumococcal challenge.

Materials and Methods

Pneumococcal Strains

All pneumococcal strains used in this study are shown in Table 1. Pneumococci were maintained as frozen stocks (−80°C) in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), with 10% glycerol. In each experiment, the isolates were plated on blood agar prior to growth in THY.

Cloning of *pspAs*, *pds* and Hybrid Genes

Gene fragments encoding the N-terminal region of *pspA* were amplified from pneumococcal strains 245/00 (PspA1) or 94/01 (PspA2) by PCR (Figure 1). The mutant detoxified Pneumolysin gene *pdT* was obtained by PCR from the pQE-30-pdT, kindly provided by Drs. Richard Malley and James Paton. Two more Ply mutants were obtained by site-directed mutagenesis, using the protocol described by Withers-Martinez *et al.* (1999). *Pd_{H367}* or *plD1* was amplified from pneumococcal strain D39 and contains one mutation on the His 367 residue, which was substituted by

Table 1. Pneumococcal strains used in this study.

Strain	Serotype	PspA Clades	Source	Reference
P69	10A	1	UFG	20
94/01	18A	2	IAL	20
245/00	14	1	IAL	20
491/00	6B	1	IAL	20
472/96	6B	1	IAL	30
A66.1	3	2	UAB	42
D39	2	2	UAB	5

IAL: Instituto Adolfo Lutz, São Paulo, Brazil.

UFG: Universidade Federal de Goiás, Goiânia, Brazil.

UAB: University of Alabama at Birmingham.

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Arg. This mutant was first described by Berry *et al.*, 1995, and retains 0.02% of the hemolytic activity of the native protein. The same mutation was inserted in the *ply* gene from strain 472/96, which contains a natural Asp-380 to Asn substitution, in a region described as involved in complement activation (Mitchell *et al.*, 1991). This second mutant, *pd_{H367R380}* or *plD2*, contains, therefore, two mutations. The primers used to obtain the mutants are listed in Table S1. The *pspA* fragments and *ply* mutant genes were inserted into pGEM-T easy vector (Promega) and fused through complementary cohesive ends added to the primers, generating three chimeric genes: *pspA1-plD1*, *pspA1-plD2*, and *pspA2-pdT*. *pspA2-pdT* was digested with the appropriate restriction endonucleases and ligated to the linearized pQE30 (QIAGEN) expression vector; *pspA1-plD1* and *pspA1-plD2* were excised from pGEM-T easy and subcloned into linearized pAE-6xHis expression vector [37].

Expression and Purification of Recombinant Proteins

The expression of rPspA2-PdT was performed in *E. coli* M15. The recombinant fragments rPspA1, rPspA2, rPID1 and rPID2, as well as the hybrids rPspA1-PID1 and rPspA1-PID2 were expressed in *E. coli* BL21DE3. All proteins include an N-terminal histidine tag added by the expression vectors *pQE* and *pAE6xHis*. Protein expression was induced in mid-log-phase cultures with 1 mM IPTG (Sigma). rPspA2-PdT, rPspA1 and rPspA2, which were expressed in the soluble form, were purified through affinity chromatography with Ni²⁺ charged chelating Sepharose resin (HisTrap Chelating HP; GE HealthCare) in an Akta Prime apparatus (GE HealthCare), as described by Goulart *et al.* [20]. The Pds fragments and hybrids rPspA1-PID1 and rPspA1-PID2 were expressed as inclusion bodies; therefore, after cell lysis, the pellets were resuspended in equilibrium buffer (Tris 50 mM, NaCl 150 mM, Imidazole 5 mM) containing 8 M urea (GE HealthCare) and submitted to refolding by slow dilution in 2 L of equilibrium buffer prior to purification. Elution was carried out with 300 mM imidazole. The purified fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dialyzed against 10 mM Tris-HCl (pH 8), 20 mM NaCl, 0.1% glycine and stored at 20°C.

Immunoblotting

The expression and purification of the hybrid proteins was confirmed by immunoblotting. Recombinant PspAs or Pds (150 ng of each) and 300 ng of each hybrid protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). The membranes containing rPspAs

Figure 1. Scheme of Proteins and Hybrids.

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and hybrid proteins or rPds and hybrid proteins were incubated with anti-rPspA or anti-Ply antisera at 1:4000 and 1:2000 dilution, respectively, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1000; Sigma). Detection was performed with an ECL kit (GE Healthcare).

Animals and Immunization

All animal experiments were approved by the Ethics Committee at Instituto Butantan, São Paulo – SP (CEUAIB), (Permit Number: 602/09). Female BALB/c mice from Faculdade de Medicina – Universidade de São Paulo (São Paulo, Brazil) were immunized subcutaneously with 3 doses of 8.8 µg of rPspA2 or rPspA1, 11.2 µg of rPdT, rPID1 or rPID2, 20 µg of co-administered proteins (rPspA2+rPdT, rPspA1+rPID1 or rPspA1+rPID2) or 20 µg of the hybrid proteins at 14-day intervals,

using sterile saline solution 0.9% with 50 µg of Al(OH)₃ as adjuvant (50 µg per mouse). The adjuvant alone in saline was used as a control. Two weeks after the last immunization, the animals were bled by retro-orbital puncture and antibody production was evaluated by ELISA. Serum samples were analyzed individually and comparison among the groups were performed using one-way ANOVA with a Tukey's Multiple Comparison Test.

Binding and Complement Deposition Assay

Pneumococcal strains bearing family 1 PspAs (Table 1) were grown in THY up to an optical density of 0.4–0.5 (corresponding to a concentration of 10⁸ CFU/ml) and harvested by centrifugation at 2000 ×g for 3 min. The pellets were washed once with PBS, resuspended in the same buffer, and incubated in the presence of heat-inactivated pooled sera from mice immunized with rPspAs,

rPds, co-administered proteins or rPspA-Pd fusions at a final concentration of 5% for 30 min at 37°C. The sera were heat-inactivated by incubation at 56°C for 30 min to destroy the activity of serum complement. After washing with PBS, the samples were incubated with 100 µL of PBS containing FITC-conjugated anti-mouse IgG (MP Biomedicals) at 1:1000 dilution on ice for 30 min in the dark. The bacteria were washed two more times with PBS, resuspended in 1% formaldehyde and analyzed by flow cytometry, using FACS Canto II (BD Biosciences). For the complement deposition assay, after incubation with antisera, the samples received 10% of BALB/c NMS (normal mouse serum) as a complement source and were incubated at 37°C for another 30 min. The samples were washed two times with PBS and incubated with FITC-conjugated anti-C3 (MP Biomedicals) at a 1:500 dilution in 100 µL of PBS. The washes, fixation and analysis were performed as previously described [20].

Opsonophagocytic Assay

The adapted opsonophagocytic assay was performed using pneumococcal strains expressing PspA clades 1 and 2 (Table 1). The bacteria were grown in THY up to mid log phase, harvested by centrifugation at 2000×g for 3 min, washed with PBS and the pellet, resuspended in opsono buffer [26]. Aliquots containing $\sim 2.5 \times 10^6$ CFU were incubated with heat inactivated antisera against the recombinant proteins alone, co-administered or the fusion proteins at 1:16 dilution at 37°C for 30 min. Sera from mice that received saline and Al(OH)₃ was used as control. After another wash with PBS, the samples were incubated with 10% NMS from BALB/c diluted in opsono buffer at 37°C for 30 min. The samples were then washed once with PBS and incubated with 4×10^5 peritoneal cells [38] diluted in opsono buffer at 37°C for 30 min with shaking (220 rpm). The reaction was stopped by incubation on ice for 1 min. Ten-fold dilutions of the samples were performed and 10 µL aliquots of each dilution were plated on blood agar plates. The plates were incubated at 37°C, with 5% CO₂ and the pneumococcal CFU recovered, counted after 18 h. Statistical analysis of the final pneumococcal counts in each group was performed by one-way ANOVA with a Tukey's Multiple Comparison Test.

Hemolysis Inhibition Assay

The recombinant Ply was expressed using *E. coli* M15 - RM 86 clone, kindly provided by Drs. Richard Malley and James Paton, and purified by affinity chromatography. The hemolysis inhibition assay was performed in 96 wells plates. 200 µL of sheep whole blood were washed 3 times with PBS and resuspended in 10 mL of PBS. The antisera produced against PspA2, PdT, PspA2+PdT or PspA2-PdT were incubated with 8 HU (hemolytic units) of Ply at 1:40 dilution at 37°C for 30 min. The antisera generated by immunization with PspA1, PID1, PID2, co-administered proteins or fused proteins PspA1-PID1 and PspA1-PID2 were incubated with 4 HU of Ply at 1:10 dilution at 37°C for 30 min. Serum from mice that received saline and Al(OH)₃ was used as control. 50 µL aliquots of 2% red blood cells were added at a final concentration of 1%, followed by incubation at 37°C for 30 min. The plates were harvested by centrifugation at 1000×g for 10 min, and the supernatant absorbance was determined at 540 nm.

Challenge

Two weeks after the last immunization the animals were challenged with 4×10^3 CFUs of *S. pneumoniae* strain A66.1 or 5×10^6 CFUs of strain 491/00 injected by the intravenous route. The mice were monitored for 15 days and the differences between the survival rates in each group were analyzed by Mann-Whitney

U test. Moribund mice or animals that developed paralysis were euthanized by CO₂ narcosis. At the endpoint, all surviving animals were euthanized.

Results

Expression and Purification of rPspA-Pd Hybrid Proteins

The gene fragments encoding rPspAs and rPds were fused with restriction enzymes and expressed in *E. coli* using vectors that add a Histidine tag to the beginning of the amino acid sequence. Both pspA gene fragments contain the N-terminal region including the proline rich region and the non-proline block; the scheme of proteins and hybrids is shown in Figure 1. The rPspA1-PID1 and rPspA1-PID2 hybrids were expressed in inclusion bodies, denatured with urea and refolded. rPspA2-PdT was expressed in soluble form. All proteins were purified through Ni²⁺-affinity chromatography and analyzed by immunoblotting using anti-rPspA1 or anti-rPspA2 and anti-rPly antibodies (Figure 2). All hybrid proteins were expressed and purified integrally and were recognized by antibodies against PspA or Ply individually.

Mouse Immunization with Hybrid Proteins Induce Levels of Antibodies Comparable to Proteins Administered Individually

Sera obtained by BALB/c immunization with rPspAs, rPds, and rPspA-Pds were quantified by ELISA against each recombinant protein. Immunization with the hybrid proteins induced antibody levels comparable to those obtained in the groups immunized with each antigen alone (Table 2).

Antibodies Generated by Mouse Immunization with Hybrid Proteins Bind to the Surface of Pneumococci Bearing Different PspAs

Sera from mice immunized with the recombinant proteins and hybrids were tested for their ability to bind onto the pneumococcal surface. Pneumococci bearing family 1 PspAs were incubated with the antisera followed by incubation with anti-mouse IgG-FITC. Antibodies generated against rPspA2-PdT were able to bind to the surface of pneumococci bearing homologous PspAs (clade 2), similarly to anti-rPspA2 or anti-rPspA2+PdT antisera (Figure 3 A and B). Interestingly, this same antiserum was able to bind with significantly higher affinity to pneumococcal strains bearing heterologous clade 1 PspAs when compared with antiserum generated against the co-administered proteins (Figure 3 G and H). Antisera generated against the other hybrids, rPspA1-PID1 and rPspA1-PID2, also revealed strong binding to pneumococcal strains, usually comparable to that observed with antisera against the co-administered proteins (Figures 3 C, E, D, I, K and L), with few exceptions (Figure 3 F and J). However, in some cases, the hybrid proteins showed a binding capacity lower than that observed for anti-PspA antisera alone (Figures 3 D, J and L). No binding was observed when anti-Pds antisera were used.

Antibodies Induced against rPspA-Pd Hybrids Induced an Increased C3 Deposition on Pneumococci Bearing Heterologous PspAs

The antisera were evaluated as to their ability to increase complement deposition on the surface of pneumococci. *S. pneumoniae* strains expressing PspA clade 1 or clade 2 were incubated with the antisera generated against the rPspA-Pd hybrids and respective controls, in the presence of a complement source, followed by incubation with anti-C3 conjugated with FITC, and these were analyzed by FACS. When we used

Figure 2. Recognition of hybrid proteins by antibodies against PspA and Pds. Recombinant proteins were separated by SDS-PAGE and transferred to PVDF membrane - A) rPspA2 and rPspA2-PdT; B) rPspA1 and rPspA1-PID1; C) rPspA1 and rPspA1-PID2; D) rPdT and rPspA2-PdT; E) rPID1 and rPspA1-PID1; and F) rPID2 and rPspA1-PID2. The membranes were incubated with anti-rPspA2 (A) anti-rPspA1 (B and C) or anti-rPly (D, E and F) followed by incubation with anti-mouse IgG conjugated with HRP. Detection was performed with an ECL kit (GE Healthcare). Molecular mass markers (kDa) are indicated on the left.
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a bacterium containing a homologous PspA, we observed that antibodies from mice immunized with co-administered proteins showed increased complement deposition when compared with anti-serum from the group immunized with rPspA alone (Figure 4–94/00 strain). However, comparable levels of C3 deposition were observed when the same bacteria was incubated with serum from the group immunized with rPspA2-PdT or the antisera induced against the co-administered proteins, which is in agreement with the results of antibody binding. On the other hand, the anti-rPspA2-PdT antibodies induced significantly higher amounts of C3 deposition on the bacterial surface, as compared to antibodies induced against the co-administered proteins for all the other strains used in this study, including those bearing PspA2 or PspA1 (Figure 4 A66.1, 245/00, P69 strains). In relation to the antisera induced against the rPspA1-PID1 and rPspA1-PID2 hybrids, no significant differences were observed in C3 deposition on pneumococci when compared with antisera generated against rPspA1 alone or the co-administered proteins, also in accordance with antibody binding (Figure S1).

In-vitro Opsonophagocytosis Mediated by Anti-hybrid Antisera is More Efficient than Antisera against Co-administered Proteins in Heterologous Strains

Since the immunization with the rPspA2-PdT hybrid induces antibodies that exhibited both a stronger binding capacity and a more pronounced C3 deposition on the bacterial surface when compared to the other groups, these antibodies were investigated for their ability to mediate the opsonophagocytosis and killing of pneumococci *in vitro*. Bacterial strains bearing PspA clade 1 or clade 2 were incubated with antisera against rPspA2, rPdT, rPspA2+rPdT and rPspA2-PdT and a complement source, followed by incubation with murine peritoneal phagocytic cells. The samples were plated and the number of CFUs recovered after 18 h were counted. When we used the pneumococcal strain 94/

00, expressing the homologous PspA clade 2, antibodies generated against the hybrid, rPspA2-PdT, promoted a significant reduction in the number of CFU recovered when compared to control or anti-rPdT anti-serum (Figure 5). This reduction was similar to that observed when incubating with anti-rPspA2 or the sera from the co-administered antigens, anti-rPspA2+rPdT (Figure 5–94/00 strain). However, when a pneumococcal strain bearing a heterologous PspA molecule (clade 1) was used, the anti-rPspA2-PdT anti-serum was more efficient in promoting the opsonophagocytic killing of the bacterium than antisera against the co-administered proteins (Figure 5–245/00 strain).

Inhibition of Ply Cytolytic Activity by Anti-pneumolysoids Antibodies

The ability of antibodies generated against PspA1, PspA2, pneumolysoids, co-administered proteins or fused proteins to inhibit the lytic effects of Pneumolysin was tested by incubation with sheep red blood cells in the presence of recombinant Ply. All antisera from formulations including pneumolysoids significantly reduced the hemolysis of red blood cells by Pneumolysin, with the exception of anti-PID1 (Figure 6). As expected, antibodies to PspA1 and 2 did not induce a significant inhibition of hemolysis (Figure 6).

Immunization with PspA-Pds Leads to an Increased Survival against Fatal Pneumococcal Challenge

The protective effect of rPspA-Pd fusions was evaluated in comparison with the isolated rPspA, rPds or the co-administered proteins, by intravenous lethal challenge with the virulent pneumococcal strains St 491/00 (Figure 7 - A) or A66.1 (Figure 7 - B and C), expressing PspA clades 1 or 2, respectively. Figure 6 shows the survival of mice up to 15 days after challenge, when the experiment was terminated. All rPspA-Pd hybrids induced higher survival rates in comparison with the control group or Pds. Furthermore, the animals immunized with the hybrids showed an increased, but not significant, survival when compared to those immunized with rPspA alone. In fact, two of the hybrids, rPspA1-PID1 and rPspA1-PID2 induced 100% protection against pneumococcal challenge. No differences in protection were observed between mice receiving the hybrids or co-administered proteins. Immunization with rPds alone, on the other hand, did not induce protection in this model of systemic infection.

Discussion

PspA and Pds have been studied as vaccine candidates against pneumococcal infections for decades, with striking success in different animal models and, in the case of rPspA, induction of antibodies with high cross-reactivity [21] and protective potential in humans [21].

Since rPspA exhibits structural and serological variability [18], it has been suggested that the inclusion of two or more fragments would be necessary in order to increase vaccine coverage. Fusion of PspA fragments of families 1 and 2 have been demonstrated to increase protection against invasive pneumococcal infection [13,39], as well as rPspA fusion or co-administration with adjuvant molecules [40,41]. Nevertheless, the width of protection could be

Table 2. Antibody levels in mice immunized with the recombinant proteins.

Coating antigen	Antisera	Antibodies concentration (μg/mL)	P value
PspA 2	PspA2	13,350	<0,01
	PspA2-PdT	13,740	<0,01
PdT	PdT	91	<0,05
	PspA2-PdT	126	<0,01
PspA1	PspA1	10,140	<0,001
	PspA1-PID1	12,530	<0,001
	PspA1-PID2	7,000	<0,01
PID1	PID1	245	<0,05
	PspA1-PID1	313	<0,01
PID2	PID2	167	<0,05
	PspA1-PID2	241	<0,01

*p values were calculated in comparison with the control group.
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S. pn* strains – PspA Clade 2** S. pn* strains – PspA Clade 2**

Figure 3. Antibody binding onto pneumococcal surface. Pneumococcal strains containing PspA2 or PspA1 were incubated with antisera from mice immunized with rPspAs, rPdTs, co-administered proteins or hybrids, followed by incubation with anti-IgG mouse conjugated with FITC and analyzed by FACS. Serum from mice that received saline/Al(OH)₃ was used as a control. The percentage of fluorescent bacteria (>10 fluorescence intensity units) was calculated for each sample. Statistical analysis was performed by one-way ANOVA with a Tukey's Multiple Comparison Test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ for treated versus control or between immunized groups, as indicated. doi:10.1371/journal.pone.0059605.g003

further extended by including a more conserved protein in the formulation. Several formulations containing more than one pneumococcal protein have been studied [14,33,42–44]. Furthermore, it has been suggested that vaccines including the same components as mixtures or fused formulations can differ in the levels of protection that they induce. A study from Lu *et al.* [42]

demonstrated protection against fatal pneumococcal infection in mice immunized with trivalent vaccine containing fusions of rPdT, rPsaA and cell wall polysaccharide, but not with the antigens mixture. The same was observed using rPspA fused or mixed with flagellin, as an adjuvant molecule [40], suggesting that fused

Figure 4. Complement deposition on pneumococcal surface in the presence of specific antibodies. Pneumococcal strains were incubated with antisera from mice immunized with rPspAs, rPdTs, co-administered proteins or hybrids and NMS as complement source. After incubation with anti-C3 mouse conjugated with FITC, the samples were analyzed by FACS. Serum from mice that received saline/Al(OH)₃ was used as a control. The median of fluorescence intensity (MFI) was calculated for each sample. Statistical analysis was performed by one-way ANOVA with a Tukey's Multiple Comparison Test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ for treated versus control or between immunized groups, as indicated. doi:10.1371/journal.pone.0059605.g004

Figure 5. Pneumococcal phagocytosis mediated by specific antibodies in the presence of complement. Pneumococcal strains bearing PspA2 (94/01 strain) or PspA1 (245/00 strain) were incubated with antisera from mice immunized with rPspA2, rPdT, rPspA2+rPdT or rPspA2-PdT and NMS as complement source, followed by incubation with mouse peritoneal phagocytes and plated on blood agar plates. CFU recovered were counted after 18 h. Statistical analysis was performed by one-way ANOVA with a Tukey's Multiple Comparison Test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for treated versus control or between immunized groups, as indicated. doi:10.1371/journal.pone.0059605.g005

antigens could be more effective than co-administered formulations.

The present work investigated the potential of rPspA-Pd fusions to protect mice against invasive pneumococcal infections. Three fusions were produced, including two rPspA fragments and three different pneumolysoids. These fusion proteins were recognized by antibodies made against both PspA and the Pneumolysoids by Western blotting, showing that both proteins in the constructs were expressed and purified in the integral form, allowing for specific antibody recognition. The antibody levels were measured by ELISA against each recombinant protein. Although PspA was more immunogenic than the Pds (inducing antibody levels around

a thousand times higher), immunization with the hybrids induced antibody levels comparable to those produced in mice immunized with each antigen alone, indicating that no antigenic competition occurred in the fusion.

Antibodies against the recombinant proteins and hybrids were evaluated for the ability to recognize and bind to intact pneumococci by FACS. Sera from mice immunized with the fusions revealed a strong binding capacity to all pneumococci tested, comparable – and in some cases superior – to that of sera against the co-administered proteins. Particularly, sera from mice immunized with rPspA2-PdT showed a stronger binding capacity to a PspA clade 1 bearing strain, when compared to the co-

Figure 6. Inhibition of hemolytic activity of Ply on red blood cells by antisera generated against recombinant and hybrids proteins. Ply was incubated with antisera and sheep red blood cells, and the supernatant absorbance was measured at 540 nm. Results are shown as percentages of the hemolytic activity in presence of sera from mice receiving saline and $Al(OH)_3$ (control). Statistical analysis was performed by one-way ANOVA with a Tukey's Multiple Comparison Test; ** $p < 0.01$; *** $p < 0.001$. doi:10.1371/journal.pone.0059605.g006

Figure 7. Mouse immunization with rPspA-Pds confers protection against pneumococcal sepsis with strains bearing heterologous PspAs. BALB/c mice immunized with 3 doses of rPspAs, rPds, co-administered or hybrid proteins were challenged with lethal doses of pneumococcal strains bearing heterologous PspA: A – Mice were challenged with 491/00 strain (PspA1); B and C – Mice were challenged with A66.1 strain (PspA2). The mice were monitored for 15 days and differences between the survival rates in each group were analyzed by Mann-Whitney U test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ for treated versus control groups. doi:10.1371/journal.pone.0059605.g007

administered proteins. The correlation between sequence identity among PspAs and antibody binding has been demonstrated in other studies, with variable results [11,13,19,20]. In general, a strong association between PspA type and the ability of induced antibodies to recognize the PspA molecules in the bacterial surface has been observed. Therefore, the significant increase in recognition of a PspA clade 1 strain by antibodies against a clade 2-containing hybrid suggests that the genetic fusion of PspA and Pd, may have a positive effect on the immune response induced against PspA.

Recent studies using cell fractionation and Western-blotting have demonstrated that Ply localizes in the pneumococcal cell wall compartment [45,46]. However, the absence of antibody recognition observed with antibodies to Pds alone suggests that this protein is not accessible to antibodies in intact pneumococci. These results were in accordance with previous studies, which suggest that Ply is not displayed on the pneumococcal surface [47,48]. Since antibodies to Pds alone do not interact with the bacterial surface, the increased binding capacity of anti-hybrid antiserum onto pneumococci bearing heterologous PspAs when compared with anti-rPspA antiserum could be explained by a possible modification in the PspA structure caused by PdT fusion, that promoted the presentation of more conserved epitopes or by an adjuvant effect of Ply [49].

Complement deposition is the key for opsonization and phagocytosis of pneumococci. Therefore, since PspA and Ply have both been shown to interact with complement components, we investigated the ability of the induced antibodies to promote C3 deposition onto the bacterial surface. In agreement with the binding results, antibodies to the rPspA2-PdT fusion protein mediated a significant enhancement in the levels of C3 deposited on the bacterial surface in relation to antibodies generated against co-administered proteins.

In order to investigate whether the produced antibodies were able to mediate the opsonophagocytosis and killing of pneumococci, bacteria were incubated in the presence of sera induced against either the recombinant proteins alone, co-administered, or the fusion proteins, and mouse peritoneal phagocytes. Corroborating with the C3 complement deposition results, serum induced against the rPspA2-PdT fusion protein was able to promote the opsonophagocytosis and killing of pneumococcal strains. Furthermore, when compared with antisera induced against the co-

administered proteins, the anti-hybrid antiserum showed a significantly increased ability to reduce the number of CFU recovered in a strain containing a heterologous PspA.

In accordance with the in vitro assays, which revealed a strong ability of antibodies against the hybrids to mediate complement deposition and phagocytic killing of bacteria, immunization with rPspA-Pd fusions protected mice against fatal challenge with pneumococcal strains bearing heterologous PspA molecules. The results also provide an insight on the mechanism responsible for protection in this model, with the induction of antibodies capable of enhancing C3 deposition on the bacterial surface, which in turn become more susceptible to phagocytic killing.

Quin et al (2007), using mutant Ply-negative pneumococci, observed that deletion of Ply did not affect blood clearance in comparison with a wild type strain. Therefore, we did not expect the Pds alone to be protective against an intravenous challenge. In fact, none of the Pds tested confer protection in this model of infection. However, anti-Pd antibodies could neutralize the lytic effects of Ply. The ability of such antibodies to mediate protective responses was evaluated through a hemolysis inhibition assay using sheep red blood cells. All formulations including Pds induced antibodies able to inhibit hemolysis by Ply, except PID1. This result indicates that antibodies against Pds may play a role in protection from *Streptococcus pneumoniae* infections by inhibiting Ply's lytic effects. The correlation between the capacity to inhibit Ply induced hemolysis and protection conferred by Pds has been confirmed by Salha et al (2012), using a detoxified mutant PlyD1 [32].

Taken together, the results suggest that PspA-Pd fusion proteins comprise a promising vaccine strategy, able to increase the immune response mediated by cross-reactive antibodies and complement deposition to heterologous strains, and to confer protection against fatal challenge.

Supporting Information

Figure S1 Complement deposition on pneumococcal surface in the presence of specific antibodies. Pneumococcal strain D39 was incubated with antisera from mice immunized with rPspA1, rPID1, co-administered proteins or PspA1-PID1 hybrid (A), or rPspA1, PID2, co-administered proteins or PspA1-PID2 hybrid (B) and NMS as complement source. After incubation with anti-C3

mouse conjugated with FITC, the samples were analyzed by FACS. Serum from mice that received saline/ $\text{Al}(\text{OH})_3$ was used as a control. The median of fluorescence intensity (MFI) is shown for each sample. (TIF)

Table S1 Oligonucleotides used in this study: sequence of the primers used for amplification of the PspA fragments and for insertion of the mutation on the Ply gene is shown.

References

- O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, et al. (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 374: 893–902.
- Singleton RJ, Hennessy TW, Bulkow LR, Hammit LL, Zulz T, et al. (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* 297: 1784–1792.
- Hsu HE, Shutt KA, Moore MR, Beall BW, Bennett NM, et al. (2009) Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. *N Engl J Med* 360: 244–256.
- Song JH, Dagan R, Klugman KP, Fritzell B (2012) The relationship between pneumococcal serotypes and antibiotic resistance. *Vaccine* 30: 2728–2737.
- Ogunniyi AD, LeMessurier KS, Graham RM, Watt JM, Briles DE, et al. (2007) Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. *Infect Immun* 75: 1843–1851.
- Berry AM, Paton JC (2000) Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect Immun* 68: 133–140.
- Quin LR, Moore QC 3rd, McDaniel LS (2007) Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice. *Infect Immun* 75: 2067–2070.
- Ren B, Szalai AJ, Hollingshead SK, Briles DE (2004) Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect Immun* 72: 114–122.
- Tu AH, Fulgham RL, McCrory MA, Briles DE, Szalai AJ (1999) Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. *Infect Immun* 67: 4720–4724.
- McDaniel LS, Ralph BA, McDaniel DO, Briles DE (1994) Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. *Microb Pathog* 17: 323–337.
- Moreno AT, Oliveira ML, Ferreira DM, Ho PL, Darrieux M, et al. (2010) Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. *Clinical and vaccine immunology: CVI* 17: 439–446.
- Roche H, Hakansson A, Hollingshead SK, Briles DE (2003) Regions of PspA/EF3296 best able to elicit protection against *Streptococcus pneumoniae* in a murine infection model. *Infect Immun* 71: 1033–1041.
- Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes AP, et al. (2007) Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. *Infect Immun* 75: 5930–5938.
- Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, et al. (2003) Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. *J Infect Dis* 188: 339–348.
- Ferreira DM, Oliveira ML, Moreno AT, Ho PL, Briles DE, et al. Protection against nasal colonization with *Streptococcus pneumoniae* by parenteral immunization with a DNA vaccine encoding PspA (Pneumococcal surface protein A). *Microb Pathog* 48: 205–213.
- Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW (2001) Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect Immun* 69: 6718–6724.
- Kono M, Hotomi M, Hollingshead SK, Briles DE, Yamanaka N (2011) Maternal immunization with pneumococcal surface protein A protects against pneumococcal infections among derived offspring. *PLoS One* 6: e27102.
- Hollingshead SK, Becker R, Briles DE (2000) Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* 68: 5889–5900.
- Csordas FC, Perciani CT, Darrieux M, Goncalves VM, Cabrera-Crespo J, et al. (2008) Protection induced by pneumococcal surface protein A (PspA) is enhanced by conjugation to a *Streptococcus pneumoniae* capsular polysaccharide. *Vaccine* 26: 2925–2929.
- Goulart C, Darrieux M, Rodriguez D, Pimenta FC, Brandileone MC, et al. (2011) Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. *Vaccine* 29: 1634–1642.
- Briles DE, Hollingshead SK, King J, Swift A, Braun PA, et al. (2000) Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *The Journal of infectious diseases* 182: 1694–1701.
- Marriott HM, Mitchell TJ, Dockrell DH (2008) Pneumolysin: a double-edged sword during the host-pathogen interaction. *Curr Mol Med* 8: 497–509.
- Marriott HM, Dockrell DH (2006) *Streptococcus pneumoniae*: the role of apoptosis in host defense and pathogenesis. *Int J Biochem Cell Biol* 38: 1848–1854.
- Srivastava A, Henneke P, Visintin A, Morse SC, Martin V, et al. (2005) The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect Immun* 73: 6479–6487.
- Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, et al. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 100: 1966–1971.
- Witzenrath M, Pache F, Lorenz D, Koppe U, Gutbier B, et al. (2011) The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol* 187: 434–440.
- Alexander JE, Lock RA, Peeters CC, Poolman JT, Andrew PW, et al. (1994) Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. *Infect Immun* 62: 5683–5688.
- Kirkham LA, Kerr AR, Douce GR, Paterson GK, Dilts DA, et al. (2006) Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. *Infect Immun* 74: 586–593.
- Ferreira DM, Areas AP, Darrieux M, Leite LC, Miyaji EN (2006) DNA vaccines based on genetically detoxified derivatives of pneumolysin fail to protect mice against challenge with *Streptococcus pneumoniae*. *FEMS Immunol Med Microbiol* 46: 291–297.
- Paton JC, Lock RA, Lee CJ, Li JP, Berry AM, et al. (1991) Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. *Infect Immun* 59: 2297–2304.
- Wu K, Zhang X, Shi J, Li N, Li D, et al. (2010) Immunization with a combination of three pneumococcal proteins confers additive and broad protection against *Streptococcus pneumoniae* infections in Mice. *Infect Immun* 78: 1276–1283.
- Salha D, Szeto J, Myers L, Claus C, Sheung A, et al. (2012) Neutralizing antibodies elicited by a novel detoxified pneumolysin derivative, PlyD1, provide protection against both pneumococcal infection and lung injury. *Infect Immun* 80: 2212–2220.
- Denoel P, Philipp MT, Doyle L, Martin D, Carletti G, et al. (2011) A protein-based pneumococcal vaccine protects rhesus macaques from pneumonia after experimental infection with *Streptococcus pneumoniae*. *Vaccine* 29: 5495–5501.
- Berry AM, Alexander JE, Mitchell TJ, Andrew PW, Hansman D, et al. (1995) Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*. *Infect Immun* 63: 1969–1974.
- Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC (2007) Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect Immun* 75: 350–357.
- Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC (2000) Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 68: 3028–3033.
- Ramos CR, Abreu PA, Nascimento AL, Ho PL (2004) A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 37: 1103–1109.
- Rodrigues DC, Cavada BS, Abreu-De-Oliveira JT, Moreira RA, Russo M (1992) Differences in Macrophage stimulation and leukocyte accumulation in response to intraperitoneal administration of Glucose/Mannose-Binding Plant Lectins. *Brazilian J Med Biol Res* 25: 823–826.
- Xin W, Li Y, Mo H, Roland KL, Curtiss R 3rd (2009) PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhimurium extend and enhance protection against *Streptococcus pneumoniae*. *Infect Immun* 77: 4518–4528.

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Author Contributions

Conceived and designed the experiments: CG DR LCCL MD. Performed the experiments: CG TRS DR WRP MD. Analyzed the data: CG DR LCCL MD. Contributed reagents/materials/analysis tools: LCCL MD. Wrote the paper: CG DR LCCL MD.

40. Nguyen CT, Kim SY, Kim MS, Lee SE, Rhee JH (2011) Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against *Streptococcus pneumoniae* infection in mice. *Vaccine* 29: 5731–5739.
41. Oliveira ML, Miyaji EN, Ferreira DM, Moreno AT, Ferreira PC, et al. (2010) Combination of pneumococcal surface protein A (PspA) with whole cell pertussis vaccine increases protection against pneumococcal challenge in mice. *PLoS One* 5: e10863.
42. Lu YJ, Forte S, Thompson CM, Anderson PW, Malley R (2009) Protection against Pneumococcal colonization and fatal pneumonia by a trivalent conjugate of a fusion protein with the cell wall polysaccharide. *Infect Immun* 77: 2076–2083.
43. Ljutic B, Ochs M, Messham B, Ming M, Dookie A, et al. (2012) Formulation, stability and immunogenicity of a trivalent pneumococcal protein vaccine formulated with aluminum salt adjuvants. *Vaccine* 30: 2981–2988.
44. Ogunniyi AD, Folland RL, Briles DB, Hollingshead SK, Paton JC (2000) Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 68: 3028–3033.
45. Price KE, Camilli A (2009) Pneumolysin localizes to the cell wall of *Streptococcus pneumoniae*. *J Bacteriol* 191: 2163–2168.
46. Price KE, Greene NG, Camilli A (2012) Export Requirements of Pneumolysin in *Streptococcus pneumoniae*. *J Bacteriol* 194: 3651–3660.
47. Berry AM, Lock RA, Hansman D, Paton JC (1989) Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infect Immun* 57: 2324–2330.
48. Balachandran P, Hollingshead SK, Paton JC, Briles DE (2001) The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. *J Bacteriol* 183: 3108–3116.
49. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, et al. (2003) Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 100: 1966–1971.

REVIEW ARTICLE

Current status and perspectives on protein-based pneumococcal vaccines

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Abstract

Despite the efforts to expand the availability of conjugate vaccines, pneumococcal diseases still pose an enormous burden worldwide. Therefore, several proteins have been investigated as alternative vaccines, alone or in combination with other antigens. With an increasing array of techniques, many of which arose from the publication of the bacterial genome, several proteins have been identified as potential vaccine candidates, and some have even progressed to clinical trials. Also, whole cell vaccines are being studied for the induction of broad ranging protective responses. Here, we briefly summarize the current knowledge on pneumococcal proteins that are being investigated as potential vaccine candidates against pneumococcal infections, and provide an insight on the future generation of protein-based vaccines against *Streptococcus pneumoniae*.

Keywords

Pneumococcal proteins, *Streptococcus pneumoniae*, vaccine

History

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Introduction

Current pneumococcal vaccines are based on either free or conjugated capsular polysaccharides (PS). However, these two vaccine categories have major disadvantages: (1) the free polysaccharide formulations fail to protect the major risk group – young children, and (2) the conjugate vaccines are based on a limited number of polysaccharides, are associated with serotype replacement, restricted coverage, and are too expensive for use in the parts of the world with the greatest need, unless heavily subsidized (Weinberger et al., 2011). These data reinforce the need for cost-effective strategies able to confer broad protection, such as protein-based vaccines.

The development of protein-based pneumococcal vaccines has been pursued for decades with some promising results. A few proteins have been investigated by classical procedures, such as PspA and other choline-binding proteins, Pneumolysin and PsaA. After the publication of the pneumococcus genome in 2001 (Hoskins et al., 2001; Tettelin et al., 2001), many new potential targets for vaccine development emerged. *In silico* screening for vaccine candidates include the identification of predicted surface proteins based on the presence of: (i) signal peptides (such as SPase I and II); (ii) choline-binding domains (which are characteristic of many pneumococcal virulence factors); (iii) sortase motifs (LPXTG); (iv) type IV pre-pilin signal sequences;

or (v) the search for homologues of known virulence proteins (Wizemann et al., 2001). Additional antigen discovery approaches include signature-tagged mutagenesis (Gieffing et al., 2008; Hava & Camilli, 2002), which provides insights into *in vivo* gene function; anti-genomics, in which a genome-based pneumococcal peptide library was screened using human sera, allowing for identification of proteins that elicit strong immune responses in the natural host (Gieffing et al., 2008); *in vivo* transcriptional analysis, which identifies genes up-regulated in specific host niches, suggesting a possible role for the respective gene products in a particular environment; and surface proteomics (Ling et al., 2004; Morscizek et al., 2008; Overweg et al., 2000). Finally, the use of a non-encapsulated inactivated whole cell formulation allows presentation of several surface exposed common proteins in their native conformation (Malley & Anderson, 2012).

Altogether, this array of techniques has yielded many potential vaccine candidates. The present work briefly summarizes the current knowledge on pneumococcal proteins that are being investigated as potential vaccine candidates against pneumococcal infections.

Proteins identified by classical approaches

PspA

Pneumococcal surface protein A is a choline-binding protein; it inhibits otherwise spontaneous classical complement activation on the pneumococcal surface (Mukerji et al., 2012; Ren et al., 2012) and inhibits killing by the bactericidal

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peptides of lactoferrin (Mirza et al., 2011; Shaper et al., 2004). Immunization with PspA has been shown to induce antibodies that recognize the surface of intact pneumococci and promote the opsonization by C3 deposition (Ochs et al., 2008; Ren et al., 2012).

Recombinant PspA fragments have also been shown to induce protective immune responses against pneumococcal colonization, lobar pneumonia and invasive infection (Arulanandam et al., 2001; Briles et al., 2003; Darrieux et al., 2007; Ferreira et al., 2010; Moreno et al., 2010; Roche et al., 2003) (Table 1). Protective immunity is elicited by the N-terminal alpha-helical sequence, especially the first and last 100 aminoacids (Arulanandam et al., 2001; Briles et al., 2003; Darrieux et al., 2007; Moreno et al., 2010; Roche et al., 2003). This later region exhibits a pattern of sequence variation that was used to classify PspA molecules into 6 clades, distributed into three families (Hollingshead et al., 2000). Protection is

also elicited by the proline-rich domain in the center of the molecule (Daniels et al., 2010; Roche et al., 2003). Although there is structural and serological variability within PspA, the fact that the three protection-eliciting regions vary independently explains in part how most PspAs are able to cross-react to some degree regardless of their PspA clade or family (Briles et al., 2000b, 2000c). Even so, the N-terminal region has been used to identify PspAs most likely to strongly cross-react with each other (Briles et al., 2000b; Darrieux et al., 2008; Goulart et al., 2011; Hollingshead et al., 2000; Vela Coral et al., 2001). Moreover, it has been shown that selection of appropriate PspA molecules as immunogens and the use of optimal adjuvants can offer increased coverage (Darrieux et al., 2008; Goulart et al., 2011; Oliveira et al., 2010). Finally, human immunization using a family 1 PspA produced antibodies able to recognize pneumococcal strains bearing both major PspA families and the five major PspA clades

Table 1. Pneumococcal proteins evaluated as vaccine candidates.

Immunogen	Immunization route	Animal models of protection	
Classical proteins			
PspA	Nasal/intraperitoneal/subcutaneous/oral	IC; Pn; Col	
Pneumolysin or pneumolysoids	Nasal/intraperitoneal/subcutaneous	IC; Pn	
Neuraminidases	Subcutaneous	IC; OM	
PspC	Nasal/subcutaneous	IC; Col	
PcpA	Subcutaneous	IC; Pn	
PsaA	Nasal/Intraperitoneal	IC; Pn	
PppA	Nasal	Col; IC	
Post-genomics proteins			
PhT	Intraperitoneal/subcutaneous	IC; Pn; Col	
PotD	Nasal/intraperitoneal/subcutaneous	IC; Col	
StkP/PcsB	Subcutaneous	IC	
Pili proteins	Intraperitoneal	IC	
PiuA and PiaA	Intraperitoneal	IC	
Immunogen	Immunization route	Animal models of protection	Reference
Other proteins			
PrtA	Subcutaneous	IC	(Wizemann et al., 2001)
Fructose–bisphosphatealdolase (FBA)	Intraperitoneal	IC	(Ling et al., 2004)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Intraperitoneal	IC	(Ling et al., 2004)
Lipoate protein ligase (Lpl)	Subcutaneous	IC	(Morsczech et al., 2008)
Caseinolytic protease (ClpP)*	Mucosal/Subcutaneous/intraperitoneal	IC; Pn	(Cao et al., 2008, Morsczech et al., 2008, Tettelin et al., 2001)
DnaJ	Nasal/intraperitoneal†	IC; Pn; Col	(Cui et al., 2011, Khan et al., 2006, Zhong et al., 2012)
R4‡	Intraperitoneal†	IC	(Cardaci et al., 2012)
Zinc metalloprotease B (ZmpB)	Mucosal	IC; Pn¶; Col	(Gong et al., 2011)
LytA	Nasal	IC; Col	(Yuan et al., 2011, Lock et al., 1992)
Putative proteinase maturation protein A (PpmA)	Intraperitoneal	–	(Gor et al., 2005)
IgA1protease (IgA1p)	Nasal	IC	(Audouy et al., 2007)
GlpO	Intraperitoneal	IC§	(Mahdi et al., 2012)
SP2108 and SP0148	Nasal	Col	(Moffitt & Malley, 2011)
AliA	Intraperitoneal	IC	(Ogunniyi et al., 2012)
SrtA	Intraperitoneal	IC	(Gianfaldoni et al., 2009)

IC: invasive challenge; Pn: pneumonia; Col: colonization; OM: otitis media.

*ClpP is a subunit of the pneumococcal caseinolytic protease ATPase;

†Intraperitoneal immunization was performed using Complete Freund's Adjuvant;

‡R4 is a recombinant fragment of the Spr1875 protein;

¶Colonization was assessed in the lungs;

§Numbers of bacteria in brain tissue were also accessed, and were significantly lower in the group injected with GlpO;

||Cholera-toxin (CT) was used as adjuvant for immunization.

(Nabors et al., 2000). This result may be explained by the natural exposure to pneumococci, which induces anti-PspA antibodies, while immunization with a recombinant PspA fragment could boost the antibody responses against these molecules for which the immune system has been primed.

Several preclinical trials utilizing live vectors, such as *Lactobacillus casei* (Campos et al., 2008) or *Salmonella enterica* serovar Typhimurium (Li et al., 2009; Shi et al., 2010), expressing PspA fragments have been evaluated in animal models and demonstrated a protective role of anti-PspA antibodies in pneumococcal infection. Furthermore, a recombinant fragment including the proline-rich region downstream of the N-terminus was recently shown to induce antibodies that bind to the surface of intact pneumococci and mediate protection against pneumococcal infection (Daniels et al., 2010; Melin et al., 2012) (Table 1).

Due to the promising preclinical results, PspA has been taken through to clinical trials in humans. Immunization of healthy volunteers with a recombinant family 1 PspA fragment induced antibodies that cross-reacted with heterologous PspA molecules and were able to passively protect mice against invasive pneumococcal challenge with strains of diverse PspA families and clades (Briles et al., 2000b; Nabors et al., 2000). Despite the encouraging immunogenicity results, efficacy trials with PspA were not conducted in part due to the early successes of PCV7 and possibly due to concerns that PspA might induce detrimental cross-reactive responses to myosin. However, cross-reactivity between PspA and myosin has never been reported in the literature, and no report of the reason the project was dropped was ever provided. Furthermore, infection with *S. pneumoniae* shows no correlation with any autoimmune-related disease, even though induction of anti-PspA antibodies occurs in normal individuals and increases upon infection (Baril et al., 2004; Rapola et al., 2000; Virolainen et al., 2000). Therefore, it seems we cannot afford to discard PspA as a protein candidate without further investigation. In fact, two recent phase I trials of

vaccines including PspA fragments have been carried out with FDA approval (Table 2).

Pneumolysin (Ply)

Pneumolysin (Ply) is a cholesterol-dependent pore forming cytotoxin expressed by virtually all pneumococcal strains (Paton, 2011). It promotes pneumococcal evasion by inhibiting the respiratory burst and, consequently, the bactericidal activity of leukocytes (reviewed in (Paton, 1996)). It also induces complement depletion during infection (Alcantara et al., 2001) and causes injury to the pulmonary endothelial and alveolar cells, as well as the expression of pro-inflammatory cytokines (reviewed in (Marriott et al., 2008)). It was shown that Ply recognition by TLR-4 activates innate immune responses to pneumococcal infection, since TLR4^{-/-} mice were more susceptible to pneumococcal infection than wild type mice (Malley et al., 2003). Furthermore, Ply is able to induce TLR-4-independent activation of the NLRP3 inflammasome contributing to host protection against pneumococcal pneumonia (Witzenrath et al., 2011).

Several groups have worked on the production of pneumolysoids (detoxified forms of Ply) via site-directed mutagenesis of one or more residues or chemical detoxification (Berry et al., 1995; Denoel et al., 2011b; Ferreira et al., 2006; Kirkham et al., 2006; Paton et al., 1991). The immunogenicity and protective effect of Ply and its detoxified mutants were evaluated in different animal models with variable results: the native protein increased survival of mice after intraperitoneal challenge, while pneumolysoids were protective in a pneumonia model of challenge (Kirkham et al., 2006) (Table 1) and reviewed in (Marriott et al., 2008). Furthermore, pre-incubation of Ply with neutralizing antibodies prevented Ply-induced lung lesions and inflammation caused by instillation of the toxin (Salha et al., 2012). However, when used in a DNA vaccine, pneumolysoids failed to protect mice against intraperitoneal challenge (Ferreira et al., 2006).

Table 2. Pneumococcal proteins evaluated in clinical trials.

Immunogen	Institute	Phase	References
Clinical trials – Proteins			
PspA	Sanofi-Pasteur	Phase I complete	(Briles et al., 2000b; Nabors et al., 2000)
PspA and PsaA	Sanofi-Pasteur/CDC	Phase I complete	Presentation by James Maleckar, Aventis
BVH3/11 V fusion protein (also called PspA and PhtB)	ID BioMedical (acquired by GSK)	Phase II	(Who, 2006)
IC-47 (PsaA, PcsB and StkP)	InterCell AG/Novartis/PATH	Phase I complete	ClinicalTrials.gov Identifier: NCT00873431
PlyD1	Netherlands Vaccine Institute/Sanofi-Pasteur	Phase I complete	(Kamthoucha et al., 2013)
PhTD/PcpA	Sanofi-Pasteur	Phase I complete	(Bologa et al., 2012)
PhTD/PcpA and PlyD1	Intern Centre for Diarrhoeal Disease Research, Bangladesh/Sanofi-Pasteur	Phase I starting in 2013	ClinicalTrial.gov identifier: NCT01764126
PhTD	GSK	Phase II complete	(Seiberling et al., 2012)
PhTD/dPly w/o or w/o PCV10	GSK	Phase II complete	EudraCT number: 2009-012701-19
PhTD/dPly/w/PCV10 w/DTPa-HBV-IPV/Hib	GSK	Phase II complete	EudraCT number: 2010-019730-27
Others			
<i>Salmonella typhimurium</i> , expressing PspA	Arizona State University/ Biodesign Institute	Phase I complete	ClinicalTrials.gov Identifier: NCT01033409
RX1 LytA ^{-/-} PdT (Non-encapsulated killed whole cell)	Boston Children's Hospital/ Instituto Butantan/PATH	Phase I ongoing	ClinicalTrials.gov Identifier: NCT01537185

The data support pneumolysoid as a strong candidate for inclusion in a pneumococcal vaccine with adjuvant properties, and combination with other pneumococcal antigens had been shown to increase the protective efficacy of these formulations, some of which have progressed to clinical trials (Kamtchoua et al., 2013) (Table 2).

Neuraminidases

Streptococcus pneumoniae expresses two types of sialidases; NanA is necessary for successful colonization of the mucosa, while NanB contributes to pneumococcal survival in the blood (Manco et al., 2006). These proteins exert their function by cleaving terminal sialic acid residues from glycoconjugates (reviewed by King et al., 2006).

Although the protective potential of NanA against invasive disease seems lower than other vaccine candidates (Lock et al., 1988), its protective effect has been demonstrated in chinchilla models of colonization (Long et al., 2004; Tong et al., 2005) and otitis media (Long et al., 2004), with induction of high antibody titers by subcutaneous immunization (Table 1). Protection against otitis media induced by anti-NanA antibodies presumably involves blockage of NanA mediated exposure of *S. pneumoniae* eukaryotic receptors in the Eustachian tube (Long et al., 2004).

In summary, data from the immunization experiments suggest that neuraminidase could be a potential vaccine candidate against otitis media due to its protective role in this host niche, with lower efficacy against invasive disease.

PspC

PspC (also known as CbpA, SpsA, PbcA or Hic) is a polymorphic surface-exposed protein with a structural organization similar to PspA, including a coiled-coil portion, a proline-rich region and a choline-binding domain (Brooks-Walter et al., 1999). PspC binds to secretory IgA and the inhibitory complement regulator factor H. While binding to FH is important to inhibit C3 deposition on the bacterial surface, interaction with secretory IgA appears to mediate pneumococcal translocation from the nasopharynx to sterile sites such as the lungs or the bloodstream (Dave et al., 2001, 2004a, 2004b). PspC displays high sequence variability, being classified into 11 clades (Iannelli et al., 2002). These differences could reflect on the extent of protection induced by immunization with PspC.

Mucosal immunization with PspC (either expressed by *Lactobacillus casei* or using the B subunit of cholera toxin – CTB as adjuvant) was protective in a murine model of colonization (Balachandran et al., 2002; Hernani M de et al., 2011) (Table 1). However, PspC-mediated protection against systemic infection is conflicting; in one model, subcutaneous immunization of CBA/N mice with PspC elicited antibodies that cross-reacted with PspA (Brooks-Walter et al., 1999) and were protective against pneumococcal sepsis (Brooks-Walter et al., 1999; Ogunniyi et al., 2001). However, in a more recent study, immunization with PspC through nasal or subcutaneous route was not able to confer protection against an intranasal challenge (Ferreira et al., 2009).

Taken together, the results indicate that PspC is an attractive vaccine candidate against colonization, with a more modest effect against invasive infection.

PcpA

Pneumococcal choline-binding protein A (PcpA) is a surface protein expressed in more than 90% of the pneumococcal isolates tested (Selva et al., 2012) and regulated by manganese concentrations. It is attached to the bacterial surface through a C-terminal choline-binding domain, while the N-terminal portion contains several leucine-rich repeats (LRR) (Sanchez-Beato et al., 1998). The structural organization of the LRR motifs provides a scaffold for protein-protein interactions suggesting a possible role for PcpA in adhesion (Seepersaud et al., 2005).

PcpA is expressed during invasive disease (in the lungs and blood), but not in the nasal mucosa, where Mn^{2+} concentrations are high. Therefore, while PcpA is considered a good vaccine candidate against invasive disease, it is not likely that this protein should elicit protection against colonization (Glover et al., 2008; Johnston et al., 2006). Confirming these assumptions, mouse immunization with PcpA has been shown to elicit protection in models of focal pneumonia and sepsis, while no protection was observed against nasal colonization (Glover et al., 2008) (Table 1). However, a more recent study demonstrated that human antibodies against PcpA are able to inhibit bacterial adhesion to lung and nasopharyngeal epithelial cells *in vitro* (Khan et al., 2012). Altogether, these results suggest that PcpA may be a strong candidate to be included in a formulation against invasive pneumococcal infections. In fact, vaccine formulations including PcpA alone or combined with PhTD have been recently tested in a phase I clinical trial and shown to be safe and immunogenic (Bologa et al., 2012). The promising results with PcpA-PhTD vaccine have prompted the evaluation of a new formulation including PlyD1 coadministered with those proteins; the clinical trial for this vaccine is scheduled to start this year (Table 2).

PsaA

Pneumococcal surface antigen A (PsaA) is a conserved lipoprotein present in all pneumococcal strains that plays several important roles in pneumococcal virulence, such as manganese transport (Dintilhac et al., 1997), resistance to oxidative stress (Tseng et al., 2002) and bacterial adhesion (Berry & Paton, 1996; McAllister et al., 2004). Systemic immunization with recombinant PsaA induced either marginal (Talkington et al., 1996) or no protection against lethal challenge (Gor et al., 2005). In contrast with its limited role in systemic infection models, the protective efficacy of PsaA against pneumococcal carriage has been demonstrated in several studies, including the use of mucosal adjuvants (Briles et al., 2000a; Pimenta et al., 2006), DNA-based formulations (Miyaji et al., 2001), co-administration with PCV7 (Whaley et al., 2010) or expression in live vectors, such as *Lactobacilli* (Oliveira et al., 2006) and *Salmonella* (Wang et al., 2010) (Table 1).

On a whole, the results indicate that PsaA is a promising vaccine candidate against carriage, but has negligible effect against systemic pneumococcal infections.

PppA

Pneumococcal protective protein A (PppA) was isolated during a search for low molecular weight proteins in PBS washes of *Streptococcus pneumoniae* that could bind to nasopharyngeal mucin; PppA was the major component in that protein mixture. Immunoelectron microscopy studies suggested it could be surface exposed, while sequence analysis demonstrated that PppA is conserved among clinical isolates of various serotypes (Green et al., 2005).

Mucosal immunization with recombinant PppA combined with different adjuvants was protective against nasopharyngeal colonization (Green et al., 2005). Moreover, *Lactococcus lactis* expressing PppA alone (Medina et al., 2008) or in association with a probiotic induced protection against lung infection (Vintini et al., 2010) and, in the first case, also against systemic infection (Medina et al., 2008). Finally, the use of *Lactobacillus* as a mucosal adjuvant for PppA has also been shown to be protective against colonization and systemic challenges, while PppA alone did not confer protection against intraperitoneal challenge. Altogether, the results indicate that PppA is a promising vaccine candidate, especially for mucosal vaccines.

Proteins identified in the post-genomics era

PhT

The PhT family of proteins were selected from the *S. pneumoniae* genome database (Adamou et al., 2001) based on the presence of hydrophobic leader sequences, characteristic of cell surface proteins (Adamou et al., 2001; Hamel et al., 2004) and includes four members: PhTA, PhTB, PhTD and PhTE, which have highly conserved sequences (Adamou et al., 2001). Although the function of these proteins has not been completely elucidated, they have been shown to inhibit complement deposition on the bacterial surface (Ogunniyi et al., 2009) and to bind zinc (Rioux et al., 2011). The low concentrations of Zn^{+2} at the mucosal sites suggest a possible role for PhT proteins during colonization (Godfroid et al., 2011). This data was further supported by a recent study showing that purified Fab antibody fragments specific to PhTD and PhTE are able to reduce pneumococcal adhesion to human airway epithelial cells (Khan & Pichichero, 2012).

The analysis of normal human sera revealed that antibodies to PhTs are naturally produced in response to pneumococcal carriage (Holmlund et al., 2009) and during invasive infections (Adamou et al., 2001). These antibodies were able to passively protect mice against lethal intranasal challenge, indicating PhTs as promising candidates for the inclusion in future protein-based pneumococcal vaccines (Godfroid et al., 2011).

PhT proteins have been shown to induce protection in animal models of nasal and lung colonization, lethal intranasal challenge and sepsis (Adamou et al., 2001; Beghetto et al., 2006; Hamel et al., 2004; Ogunniyi et al., 2007; Wizemann et al., 2001; Zhang et al., 2001), which in some cases was superior to protection conferred by PspA and CbpA (Godfroid et al., 2011; Table 1). PhT fragments, including the N- or C-terminal regions, have also been shown to elicit protective immunity with a higher degree of cross-protection observed after immunization with the C-terminus

(Adamou et al., 2001). Comparisons amongst the four PhTs indicate PhTD as the most protective protein in this family (Adamou et al., 2001) (Table 1).

The encouraging preliminary studies have prompted the investigation of PhTs co-administered or in fusion with other pneumococcal proteins, with some formulations progressing to phase I clinical trials (Table 2).

PotD

PotD is a member of the polyamine transport operon, which consists of four members, *potABCD* (Hoskins et al., 2001). It is a surface-associated (Shah et al., 2006), polyamine-binding protein, mediating polyamine intake by the bacterial cell (Ware et al., 2005). Signature tagged mutagenesis studies have suggested a role for PotD in pneumococcal virulence (Polissi et al., 1998). This result was confirmed by evaluation of PotD negative mutant pneumococci, which displayed reduced virulence in mouse models of sepsis and pneumonia.

Subcutaneous immunization with recombinant PotD was able to protect mice from fatal pneumococcal challenge. Passive immunization with sera from immunized rabbits elicited protection in mice against intraperitoneal infection (Shah & Swiatlo, 2006). Nasal immunization with PotD has also been shown to reduce mucosal colonization with *S. pneumoniae* (Shah et al., 2008) (Table 1).

The requirement of PotD for normal pneumococcal growth, its effect on virulence in the human host and ability to elicit protection in mucosal and systemic animal models render this protein an interesting candidate to be considered for inclusion in protein-based vaccines.

StkP and PcsB

Serine/threonine protein kinase (StkP) and Protein required for cell wall separation of group B streptococcus (PcsB) are two conserved proteins identified by the ANTIGENome strategy, in which a pneumococcal peptide library was screened using sera from exposed or convalescing patients (Giefing et al., 2008). After a series of *in vitro* tests and animal studies, StkP and PcsB were shown to be cross-protective in models of sepsis and pneumonia (Giefing et al., 2008). Sequence analysis revealed that these proteins are highly conserved among pneumococcal strains (Giefing et al., 2008).

StkP has been shown to act as a global regulator of gene expression in *S. pneumoniae*, including oxidative stress response, iron uptake, DNA repair, pyrimidine biosynthesis and cell wall metabolism (Saskova et al., 2007). This functional diversity, together with a surface location (Giefing et al., 2008, 2010), suggests that StkP is an interesting candidate to be included in future pneumococcal vaccines. PcsB is a hydrolase involved in cell wall separation, which is also accessible to antibodies (Mills et al., 2007).

StkP and PcsB have been successfully tested in animal models of pneumococcal infection, eliciting protection against fatal intraperitoneal and intranasal challenges and lobar pneumonia with different pneumococcal strains (Giefing et al., 2008) (Table 1). In face of the promising results in mice, StkP and PcsB were included in a formulation

with PsaA, which was evaluated in a phase I clinical trial and shown to induce an increase in specific antibodies against all three proteins in the vaccinated individuals (Table 2 and Nagy, personal communication).

Pili proteins

The pneumococcal pilus was first characterized in the serotype 4 TIGR4 strain (Barocchi et al., 2006) and is composed of three structural proteins named RrgA, RrgB and RrgC (Barocchi et al., 2006; Hilleringmann et al., 2009). RrgB forms the backbone of the pilus structure (Spraggon et al., 2010), while RrgA and RrgC are accessory proteins located on the external and internal ends, respectively (Hilleringmann et al., 2009). RrgA is an adhesin involved in biofilm formation. RrgB occurs in three forms, and a hybrid protein including a fusion protein with all three variants has been shown to be protective against systemic challenge with different pilated pneumococcal strains (Harfouche et al., 2012; Wizemann et al., 2001) (Table 1).

Mouse immunization with the recombinant pilus subunits, alone or in combination, was able to reduce bacteremia and protect against fatal systemic infection (Gianfaldoni et al., 2007). Passive immunization with antibodies raised against the combined proteins was also able to reduce bacterial load in the blood of mice (Gianfaldoni et al., 2007). However, the pilus is present in no more than one third of the pneumococcal strains tested (Paterson & Mitchell, 2006; Selva et al., 2012) and exhibit a biphasic expression pattern (De Angelis et al., 2011). The limited distribution and variability of pilus proteins could offer reduced coverage if they were used solely as components of pneumococcal vaccines. However, their ability to confer protection in mice against different types of pneumococcal infection makes pili proteins promising candidates for inclusion in multi-component vaccine formulations.

PiuA and PiaA

PiuA and PiaA are two components of the iron uptake system that have been identified as possible lipoproteins located in the pneumococcal membrane (Brown et al., 2001b) and are required for full virulence in animal models of pneumococcal infection (Brown et al., 2001a). PCR analysis has demonstrated that both genes are present in all pneumococci tested (Brown et al., 2001b) and show high sequence similarities to surface iron receptors from other organisms.

Antibodies to PiuA and PiaA have been found in healthy infants, and were increased in the serum of convalescent septicemia patients in comparison with the acute-phase of disease (Whalan et al., 2005). Mouse immunization with recombinant PiuA or PiaA induced antibodies that cross-reacted with each other and were protective against systemic challenge with virulent pneumococci, with the highest protection levels being observed in the group injected with the two proteins combined (Brown et al., 2001b). The role of antibodies in this infection model was further emphasized in passive protection experiments (Brown et al., 2001b; Jomaa et al., 2006, 2005), and demonstrated to function primarily by enhancing bacterial phagocytosis rather than interfering with iron transportation (Jomaa et al., 2005).

Multicomponent protein-based vaccines

Classic well-studied virulence factors, such as PspA, pneumolysoids and PsaA have been combined amongst each other or with newly discovered protein candidates in order to obtain increased immunogenicity and/or broader protective efficacy. The combination of PspA with antigens such as the pneumolysoid, PdB, or a mixture of antigens (PdB, PspC, ClpP and/or PhT proteins) was able to increase protection in different challenge models (Cao et al., 2007; Ogunniyi et al., 2000, 2007).

Different pneumolysoids have been co-administered with antigens such as CbpA or ClpP and Lpl, increasing either protective potency or coverage (Ogunniyi et al., 2001; Wu et al., 2010). A chemically detoxified pneumolysoid combined with PhTD was recently shown to be protective in rhesus macaques following a pneumococcal challenge (Denoel et al., 2011b). PhTD was also tested in mice with promising results in combination with PS 1 and 3 (Denoel et al., 2011a; Table 1).

Formulations including PhTD coadministered with PcpA have been evaluated in a phase I clinical trial, and proven to be immunogenic and safe in humans (Bologa et al., 2012). A new phase 1 clinical trial is currently being performed using PhTD, PcpA and PlyD1 (Table 2).

Another preclinical assay using a combination of glutamyl tRNA synthetase (Gts), PotD and sortase A (SrtA), showed increased ability to protect mice against pneumococcal colonization and sepsis (Min et al., 2012).

In the combination of PcsB, StkP and PsaA using alum or IC31 as adjuvants, the importance of the adjuvant in the protective immunity induced was clear (Olafsdottir et al., 2012), suggesting that the choice of adjuvant will have an impact even in multicomponent vaccines.

Whole cell pneumococcal vaccine

The whole cell pneumococcal vaccine (WCV), is an inactivated cellular preparation of a non-encapsulated strain of *S. pneumonia* derived from R × 1, in which the *lytA* gene was deleted and the *ply* gene was substituted for *pdT*; this formulation presents a combination of protective antigens common to all strains (Malley et al., 2001). Initially it was proposed that this preparation would be administered intranasally with a strong mucosal adjuvant, inducing antibody-independent, CD4+ T cell-dependent immunity, with production of IL-17, leading to the accelerated clearance of pneumococci from the nasopharynx (Malley et al., 2005). This protection against intranasal colonization was shown to be effective against multiple serotypes of pneumococci (Malley et al., 2004).

More recently, it was shown that systemic administration of the WCV preparation adsorbed to aluminum salts, in addition to inducing IL-17 mediated protection against intranasal challenge, also induces antibody formation that protects mice in a model of lethal aspiration pneumonia, which is also independent of CD4+ T cells (Lu et al., 2010). Priming of mice with the WCV and screening for humoral and cellular immune responses against a panel of clinical isolates from invasive disease and carriage with different serotypes, suggested that the WCV would provide functional broad

coverage against a range of serotypes (Malley & Anderson, 2012). The WCV is currently in Phase 1 clinical trials in the United States (Table 2).

Chimeric protein-based pneumococcal vaccines

Fusions of pneumococcal proteins to other proteins or to polysaccharides have been proposed in many studies with stimulating results. The main advantages of this approach include the possibility of immune modulation, as well as the enhancement in immunogenicity, possibly by exposure of hidden epitopes, or even reduction in production costs, since these formulations can be purified as single antigens. The following paragraphs summarize some of the results with antigen fusions or conjugations.

Due to the structural and serological variability among PspA molecules, hybrid proteins including fragments of the predominant families were obtained, demonstrating extended protection mediated by complement deposition on the bacterial surface (Darrieux et al., 2007). The fusion of PspA with the TLR5 agonist, flagellin, demonstrated its potential as a mucosal vaccine (Nguyen et al., 2011). Recently, PspA molecules were fused to different pneumolysoids, showing increased ability to promote cross-reactive antibodies and enhance complement deposition on the bacterial surface, conferring protection against fatal challenge in mice (Goulart et al., 2013).

Different pneumococcal proteins have been evaluated as protein carriers for pneumococcal polysaccharide (PS). Pneumolysoids, such as PdB, PdT or even a fusion of PdT with PsaA, have been conjugated with PSs, inducing opsonizing antibodies against the PS, and in some cases protection against challenge (Lu et al., 2009; Michon et al., 1998). Different PspAs have also been used as carrier proteins for different PS, generating a variety of conjugates with promising results. Protective immune responses were induced against both antigens of the conjugates with high ability to bind onto the pneumococcal surface and induce C3 deposition, including opsonophagocytic antibodies, which protected mice against pneumococcal challenge (Csordas et al., 2008; Perciani et al., 2013; Santamaria et al., 2011). On a whole, it seems as the strategy of fusing or conjugating antigens increases their immunogenicity and protective potency.

Will recombinant proteins be immunogenic enough?

Considering the vaccines that are effective in humans, they can be divided into two major classes: (1) the live, attenuated viruses and bacteria, such as oral Polio or BCG vaccines, or the whole cell inactivated microorganisms, such as the whole cell Pertussis vaccine, those which resemble an acute infection and (2) highly immunogenic molecules, such as the toxin derivatives and polysaccharides, as for example the Tetanus and Diphtheria vaccines, and *Streptococcus* and *Haemophilus* B vaccines, which can be considered special cases of antigens. In the second class, there are also the virus-like particles (VLPs), such as the Hepatitis B and Herpes Virus (HPV) vaccines, which are again very special cases of recombinant protein antigens, where the immunogenic target is multiplied many-fold and they also resemble viruses. These are actually the only successful cases of vaccines.

There has been an enormous amount of assays investigating different recombinant proteins as vaccine candidates against the most varied pathogens with very limited success so far. In the case of the malaria vaccine, the circumsporozoite surface protein (CSP) antigen has been tested in many different presentations and formulations, but only became partially protective in humans recently, when presented as a VLP (in a Hepatitis B vaccine backbone), together with a very potent adjuvant (Agnandji et al., 2011, 2012). In the case of the meningococcal B reverse vaccinology-derived vaccine, a combination of recombinant proteins only became sufficiently immunogenic and protective in humans when combined with the respective outer membrane vesicle (OMV) (Santolaya et al., 2012). These two examples may be suggesting that common recombinant proteins per se may not be immunogenic enough to induce a protective immune response in humans.

There are many proteins and protein-based vaccines that have been assayed as vaccine candidates against pneumococcus. Most of them have not progressed past the preclinical trials in mice (Table 1). Some proteins have been better characterized and even progressed into phase I trials in humans (Table 2). However, the immunogenicity in humans has been low for most of them. This may indicate that more immunogenic presentation systems may be required. The presentation of the same proteins as VLPs may increase their immunogenicity, although it is not an easy system to work with. Alternatively, coupling or conjugating the proteins to something larger and more immunogenic, such as a polysaccharide or an OMV, may also improve their presentation (Csordas et al., 2008; Santolaya et al., 2012). The use of adjuvants will certainly play a role in increasing the immunogenicity of protein formulations (Olafsdottir et al., 2012; Oliveira et al., 2010). Alternatively, expression in a live attenuated microorganism may also enhance the protein's immunogenicity, but will bring all the complexity of the systems themselves (Langermann et al., 1994; Li et al., 2009).

In this sense, a whole cell vaccine may have an advantage, presenting many antigens in their natural conformation (Moffitt et al., 2012), although it will remain to be shown if an inactivated formulation presenting small quantities of several antigens will be immunogenic enough to be protective in humans. Another important issue that needs to be addressed regards the endpoint targeted by the vaccine formulation. While prevention of invasive diseases and pneumonia are the ultimate goal, vaccines that prevent nasopharyngeal colonization will impact on disease onset, and also limit pneumococcal spread by herd immunity. The choice of the target response will impact on the size of the clinical assay, its cost and availability of locations with infrastructure to perform this type of assay. Although colonization is not a direct measure of disease impact, it is a necessary step for disease progression. With the increasing implementation of PCVs it is becoming evident that the most convenient endpoint for clinical assays of protein vaccines will probably be colonization. Models for evaluating experimental human carriage have been proposed (McCool et al., 2002; Wright et al., 2012), and demonstrated a specific and serotype independent immune response in volunteers that had been challenged but were not colonized.

It is important to note, however, that the presence of a rich bacterial flora on the nasopharynx raises concern as to whether pneumococcal reduction through widespread vaccination will lead to disease replacement by other potentially pathogenic species (reviewed by Weiser, 2010). Therefore, this aspect should be included in the analysis of vaccine effect.

Altogether, these results suggest that an effective pneumococcal vaccine will probably require the inclusion of multiple proteins with high immunogenicity and broad coverage. However, even in those combined formulations, the immunogenicity of plain recombinant proteins may not be sufficient to achieve protective immunity in humans, and it will probably be necessary to use more immunogenic presentations/adjuvants than those currently being used.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

- Adamou JE, Heinrichs JH, Erwin AL, et al. (2001). Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infection and immunity* 69:949–58.
- Agnandji ST, Lell B, Soulanoudjingar SS, et al. (2011). First results of phase 3 trial of Rts,S/AS01 malaria vaccine in African children. *N Engl J Med* 365:1863–75.
- Agnandji ST, Lell B, Fernandes JF, et al. (2012). A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. *N Engl J Med* 367: 2284–95.
- Alcantara RB, Preheim LC, GENTRY-Nielsen MJ. (2001). Pneumolysin-induced complement depletion during experimental pneumococcal bacteremia. *Infect Immun* 69:3569–75.
- Arulanandam BP, Lynch JM, Briles DE, et al. (2001). Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect Immun* 69: 6718–24.
- Audouy SA, van Selm S, van Roosmalen ML, et al. (2007). Development of lactococcal GEM-based pneumococcal vaccines. *Vaccine* 25: 2497–506.
- Balachandran P, Brooks-Walter A, Virolainen-Julkunen A, et al. (2002). Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. *Infect Immun* 70:2526–34.
- Baril L, Briles DE, Crozier P, et al. (2004). Characterization of antibodies to PspA and PsaA in adults over 50 years of age with invasive pneumococcal disease. *Vaccine* 23:789–93.
- Barocchi MA, Ries J, Zogaj X, et al. (2006). A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci U S A* 103:2857–62.
- Beghetto E, Gargano N, Ricci S, et al. (2006). Discovery of novel *Streptococcus pneumoniae* antigens by screening a whole-genome lambda-display library. *FEMS Microbiol Lett* 262:14–21.
- Berry AM, Alexander JE, Mitchell TJ, et al. (1995). Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*. *Infect Immun* 63:1969–74.
- Berry AM, Paton JC. (1996). Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infect Immun* 64:5255–62.
- Bologa M, Kamtchoua T, Hopfer R, et al. (2012). Safety and immunogenicity of pneumococcal protein vaccine candidates: monovalent choline-binding protein A (PcpA) vaccine and bivalent PcpA-pneumococcal histidine triad protein D vaccine. *Vaccine* 30:7461–8.
- Briles DE, Ades E, Paton JC, et al. (2000a). Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect Immun* 68:796–800.
- Briles DE, Hollingshead SK, King J, et al. (2000b). Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J Infect Dis* 182:1694–701.
- Briles DE, Hollingshead SK, Nabors GS, et al. (2000c). The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. *Vaccine* 19:S87–95.
- Briles DE, Hollingshead SK, Paton JC, et al. (2003). Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. *J Infect Dis* 188:339–48.
- Brooks-Walter A, Briles DE, Hollingshead SK. (1999). The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, Pspc, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infect Immun* 67:6533–42.
- Brown JS, Gilliland SM, Holden DW. (2001a). A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* 40:572–85.
- Brown JS, Ogunniyi AD, Woodrow MC, et al. (2001b). Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infect Immun* 69:6702–6.
- Campos IB, Darrieux M, Ferreira DM, et al. (2008). Nasal immunization of mice with *Lactobacillus casei* expressing the Pneumococcal Surface Protein A: induction of antibodies, complement deposition and partial protection against *Streptococcus pneumoniae* challenge. *Microbes Infect* 10:481–8.
- Cao J, Chen D, Xu W, et al. (2007). Enhanced protection against pneumococcal infection elicited by immunization with the combination of PspA, Pspc, and ClpP. *Vaccine* 25:4996–5005.
- Cao J, Chen T, Li D, et al. (2008). Mucosal immunization with purified ClpP could elicit protective efficacy against pneumococcal pneumonia and sepsis in mice. *Microbes Infect* 10:1536–42.
- Cardaci A, Papasergi S, Midiri A, et al. (2012). Protective activity of *Streptococcus pneumoniae* Spr1875 protein fragments identified using a phage displayed genomic library. *PLoS One* 7: e36588.
- Csordas FC, Perciani CT, Darrieux M, et al. (2008). Protection induced by pneumococcal surface protein A (PspA) is enhanced by conjugation to a *Streptococcus pneumoniae* capsular polysaccharide. *Vaccine* 26:2925–9.
- Cui Y, Zhang X, Gong Y, et al. (2011). Immunization with DnaJ (hsp40) could elicit protection against nasopharyngeal colonization and invasive infection caused by different strains of *Streptococcus pneumoniae*. *Vaccine* 29:1736–44.
- Daniels CC, Coan P, King J, et al. (2010). The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. *Infect Immun* 78:2163–72.
- Darrieux M, Miyaji EN, Ferreira DM, et al. (2007). Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. *Infect Immun* 75: 5930–8.
- Darrieux M, Moreno AT, Ferreira DM, et al. (2008). Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *J Med Microbiol* 57:273–8.
- Dave S, Brooks-Walter A, Pangburn MK, Mcdaniel LS. (2001). Pspc, a pneumococcal surface protein, binds human factor H. *Infect Immun* 69:3435–7.
- Dave S, Carmicle S, Hammerschmidt S, et al. (2004a). Dual roles of Pspc, a surface protein of *Streptococcus pneumoniae*, in binding human secretory IgA and factor H. *J Immunol* 173:471–7.
- Dave S, Pangburn MK, Pruitt C, Mcdaniel LS. (2004b). Interaction of human factor H with PspC of *Streptococcus pneumoniae*. *Indian J Med Res* 119:66–73.
- de Angelis G, Moschioni M, Muzzi A, et al. (2011). The *Streptococcus pneumoniae* pilus-1 displays a biphasic expression pattern. *PLoS One* 6:e21269.
- Denol P, Godfroid F, Hermand P, et al. (2011a). Combined protective effects of anti-PhTD and anti-pneumococcal polysaccharides. *Vaccine* 29:6451–3.
- Denol P, Philipp MT, Doyle L, et al. (2011b). A protein-based pneumococcal vaccine protects rhesus macaques from pneumonia

- after experimental infection with *Streptococcus pneumoniae*. *Vaccine* 29:5495–501.
- Dintilhac A, Alloing G, Granadel C, Claverys JP. (1997). Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol Microbiol* 25:727–39.
- Ferreira DM, Areas AP, Darrieux M, et al. (2006). DNA vaccines based on genetically detoxified derivatives of pneumolysin fail to protect mice against challenge with *Streptococcus pneumoniae*. *FEMS Immunol Med Microbiol* 46:291–7.
- Ferreira DM, Darrieux M, Silva DA, et al. (2009). Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. *Clin Vaccine Immunol* 16:636–45.
- Ferreira DM, Oliveira ML, Moreno AT, et al. (2010). Protection against nasal colonization with *Streptococcus pneumoniae* by parenteral immunization with a DNA vaccine encoding PspA (Pneumococcal surface protein A). *Microb Pathog* 48:205–13.
- Gianfaldoni C, Censini S, Hillerlingmann M, et al. (2007). *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. *Infect Immun* 75:1059–62.
- Gianfaldoni C, Maccari S, Pancotto L, et al. (2009). Sortase A confers protection against *Streptococcus pneumoniae* in mice. *Infect Immun* 77:2957–61.
- Giefing C, Jelencsics KE, Gelbmann D, et al. (2010). The pneumococcal eukaryotic-type serine/threonine protein kinase StkP co-localizes with the cell division apparatus and interacts with FtsZ *in vitro*. *Microbiology* 156:1697–707.
- Giefing C, Meinke AL, Hanner M, et al. (2008). Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. *J Exp Med* 205:117–31.
- Glover DT, Hollingshead SK, Briles DE. (2008). *Streptococcus pneumoniae* surface protein PcpA elicits protection against lung infection and fatal sepsis. *Infect Immun* 76:2767–76.
- Godfroid F, Hermand P, Verlant V, et al. (2011). Preclinical evaluation of the Pht proteins as potential cross-protective pneumococcal vaccine antigens. *Infect Immun* 79:238–45.
- Gong Y, Xu W, Cui Y, et al. (2011). Immunization with a ZmpB-based protein vaccine could protect against pneumococcal diseases in mice. *Infect Immun* 79:867–78.
- Gor DO, Ding X, Briles DE, et al. (2005). Relationship between surface accessibility for Ppma, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae*. *Infect Immun* 73:1304–12.
- Goulart C, DA Silva TR, Rodriguez D, et al. (2013). Characterization of protective immune responses induced by pneumococcal surface protein A in fusion with pneumolysin derivatives. *PLoS One* 8:e59605.
- Goulart C, Darrieux M, Rodriguez D, et al. (2011). Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. *Vaccine* 29:1634–42.
- Green BA, Zhang Y, Masi AW, et al. (2005). Pppa, a surface-exposed protein of *Streptococcus pneumoniae*, elicits cross-reactive antibodies that reduce colonization in a murine intranasal immunization and challenge model. *Infect Immun* 73:981–9.
- Hamel J, Charland N, Pineau I, et al. (2004). Prevention of pneumococcal disease in mice immunized with conserved surface-accessible proteins. *Infect Immun* 72:2659–70.
- Harfouche C, Filippini S, Gianfaldoni C, et al. (2012). RrgB321, a fusion protein of the three variants of the pneumococcal pilus backbone RrgB, is protective *in vivo* and elicits opsonic antibodies. *Infect Immun* 80:451–60.
- Hava DL, Camilli A. (2002). Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Molecular microbiology* 45:1389–406.
- Hernani M de L, Ferreira PC, Ferreira DM, et al. (2011). Nasal immunization of mice with *Lactobacillus casei* expressing the pneumococcal surface protein C primes the immune system and decreases pneumococcal nasopharyngeal colonization in mice. *FEMS Immunol Med Microbiol* 62:263–72.
- Hilleringmann M, Ringler P, Muller SA, et al. (2009). Molecular architecture of *Streptococcus pneumoniae* TIGR4 pili. *EMBO J* 28:3921–30.
- Hollingshead SK, Becker R, Briles DE. (2000). Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* 68:5889–900.
- Holmlund E, Quiambao B, Ollgren J, et al. (2009). Antibodies to pneumococcal proteins PhtD, CbpA, and LytC in Filipino pregnant women and their infants in relation to pneumococcal carriage. *Clin Vaccine Immunol* 16:916–23.
- Hoskins J, Alborn JR WE, Arnold J, et al. (2001). Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol* 183:5709–17.
- Iannelli F, Oggioni MR, Pozzi G. (2002). Allelic variation in the highly polymorphic locus *pspC* of *Streptococcus pneumoniae*. *Gene* 284:63–71.
- Johnston JW, Briles DE, Myers LE, Hollingshead SK. (2006). Mn2+-dependent regulation of multiple genes in *Streptococcus pneumoniae* through PsaR and the resultant impact on virulence. *Infect Immun* 74:1171–80.
- Jomaa M, Terry S, Hale C, et al. (2006). Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with *Streptococcus pneumoniae*. *Vaccine* 24:5133–9.
- Jomaa M, Yuste J, Paton JC, et al. (2005). Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun* 73:6852–9.
- Kamtchoua T, Bologna M, Hopfer R, et al. (2013). Safety and immunogenicity of the pneumococcal pneumolysin derivative PlyD1 in a single-antigen protein vaccine candidate in adults. *Vaccine* 31:327–33.
- Khan MN, Bansal A, Shukla D, et al. (2006). Immunogenicity and protective efficacy of DnaJ (hsp40) of *Streptococcus pneumoniae* against lethal infection in mice. *Vaccine* 24:6225–31.
- Khan MN, Pichichero ME. (2012). Vaccine candidates PhtD and PhtE of *Streptococcus pneumoniae* are adhesins that elicit functional antibodies in humans. *Vaccine* 30:2900–7.
- Khan MN, Sharma SK, Filkins LM, Pichichero ME. (2012). PcpA of *Streptococcus pneumoniae* mediates adherence to nasopharyngeal and lung epithelial cells and elicits functional antibodies in humans. *Microbes Infect* 14:1102–10.
- King SJ, Hippe KR, Weiser JN. (2006). Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. *Mol Microbiol* 59:961–74.
- Kirkham LA, Kerr AR, Douce GR, et al. (2006). Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. *Infect Immun* 74:586–93.
- Langermann S, Palaszynski SR, Burlein JE, et al. (1994). Protective humoral response against pneumococcal infection in mice elicited by recombinant bacille Calmette-Guerin vaccines expressing pneumococcal surface protein A. *J Exp Med* 180:2277–86.
- Li Y, Wang S, Scarpellini G, et al. (2009). Evaluation of new generation *Salmonella enterica* serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. *Proc Natl Acad Sci U S A* 106:593–8.
- Ling E, Feldman G, Portnoi M, et al. (2004). Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. *Clin Exp Immunol* 138:290–8.
- Lock RA, Hansman D, Paton JC. (1992). Comparative efficacy of autolysin and pneumolysin as immunogens protecting mice against infection by *Streptococcus pneumoniae*. *Microb Pathog* 12:137–43.
- Lock RA, Paton JC, Hansman D. (1988). Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. *Microb Pathog* 5:461–7.
- Long JP, Tong HH, Demaria TF. (2004). Immunization with native or recombinant *Streptococcus pneumoniae* neuraminidase affords protection in the chinchilla otitis media model. *Infect Immun* 72:4309–13.
- Lu YJ, Forte S, Thompson CM, et al. (2009). Protection against pneumococcal colonization and fatal pneumonia by a trivalent conjugate of a fusion protein with the cell wall polysaccharide. *Infect Immun* 77:2076–83.
- Lu YJ, Leite L, Goncalves VM, et al. (2010). GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. *Vaccine* 28:7468–75.

- Mahdi LK, Wang H, Van der Hoek MB, et al. (2012). Identification of a novel pneumococcal vaccine antigen preferentially expressed during meningitis in mice. *J Clin Invest* 122:2208–20.
- Malley R, Anderson PW. (2012). Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity. *Proc Natl Acad Sci U S A* 109:3623–7.
- Malley R, Henneke P, Morse SC, et al. (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 100:1966–71.
- Malley R, Lipsitch M, Stack A, et al. (2001). Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infect Immun* 69:4870–3.
- Malley R, Morse SC, Leite LC, et al. (2004). Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. *Infect Immun* 72:4290–2.
- Malley R, Trzcinski K, Srivastava A, et al. (2005). CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc Natl Acad Sci U S A* 102:4848–53.
- Manco S, Hernon F, Yesilkaya H, et al. (2006). Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. *Infect Immun* 74:4014–20.
- Marriott HM, Mitchell TJ, Dockrell DH. (2008). Pneumolysin: a double-edged sword during the host-pathogen interaction. *Curr Mol Med* 8:497–509.
- Mcallister LJ, Tseng HJ, Ogunniyi AD, et al. (2004). Molecular analysis of the psa permease complex of *Streptococcus pneumoniae*. *Mol Microbiol* 53:889–901.
- Mccool TL, Cate TR, Moy G, Weiser JN. (2002). The immune response to pneumococcal proteins during experimental human carriage. *J Exp Med* 195:359–65.
- Medina M, Villena J, Vintini E, et al. (2008). Nasal immunization with *Lactococcus lactis* expressing the pneumococcal protective protein A induces protective immunity in mice. *Infect Immun* 76:2696–705.
- Melin M, Coan P, Hollingshead S. (2012). Development of cross-reactive antibodies to the proline-rich region of pneumococcal surface protein A in children. *Vaccine* 30:7157–60.
- Michon F, Fusco PC, Minetti CA, et al. (1998). Multivalent pneumococcal capsular polysaccharide conjugate vaccines employing genetically detoxified pneumolysin as a carrier protein. *Vaccine* 16:1732–41.
- Mills MF, Marquart ME, Mcdaniel LS. (2007). Localization of PcsB of *Streptococcus pneumoniae* and its differential expression in response to stress. *J bacteriol* 189:4544–6.
- Min X, Zhang X, Wang H, et al. (2012). Protection against pneumococcal infection elicited by immunization with glutamyl tRNA synthetase, polyamine transport protein D and sortase A. *Vaccine* 30:3624–33.
- Mirza S, Wilson L, Benjamin JR. WH, et al. (2011). Serine protease PrtA from *Streptococcus pneumoniae* plays a role in the killing of *S. pneumoniae* by apolactoferrin. *Infect Immun* 79:2440–50.
- Miyaji EN, Dias WO, Gamberini M, et al. (2001). PsaA (pneumococcal surface adhesin A) and PspA (pneumococcal surface protein A) DNA vaccines induce humoral and cellular immune responses against *Streptococcus pneumoniae*. *Vaccine* 20:805–12.
- Moffitt KL, Malley R. (2011). Next generation pneumococcal vaccines. *Curr Opin Immunol* 23:407–13.
- Moffitt KL, Yadav P, Weinberger DM, et al. (2012). Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. *Vaccine* 30:4316–22.
- Moreno AT, Oliveira ML, Ferreira DM, et al. (2010). Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. *Clin Vaccine Immunol* 17:439–46.
- Morszczek C, Prokhorova T, Sigh J, et al. (2008). *Streptococcus pneumoniae*: proteomics of surface proteins for vaccine development. *Clin Microbiol Infect* 14:74–81.
- Mukerji R, Mirza S, Roche AM, et al. (2012). Pneumococcal surface protein a inhibits complement deposition on the pneumococcal surface by competing with the binding of C-reactive protein to cell-surface phosphocholine. *J Immunol* 189:5327–35.
- Nabors GS, Braun PA, Herrmann DJ, et al. (2000). Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine* 18:1743–54.
- Nguyen CT, Kim SY, Kim MS, et al. (2011). Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against *Streptococcus pneumoniae* infection in mice. *Vaccine* 29:5731–9.
- Ochs MM, Bartlett W, Briles DE, et al. (2008). Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. *Microb Pathog* 44:204–14.
- Ogunniyi AD, Folland RL, Briles DE, et al. (2000). Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect Immun* 68:3028–33.
- Ogunniyi AD, Grabowicz M, Briles DE, et al. (2007). Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect Immun* 75:350–7.
- Ogunniyi AD, Grabowicz M, Mahdi LK, et al. (2009). Pneumococcal histidine triad proteins are regulated by the Zn2+-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. *FASEB J* 23:731–8.
- Ogunniyi AD, Mahdi LK, Trappetti C, et al. (2012). Identification of genes that contribute to the pathogenesis of invasive pneumococcal disease by *in vivo* transcriptomic analysis. *Infect Immun* 80:3268–78.
- Ogunniyi AD, Woodrow MC, Poolman JT, Paton JC. (2001). Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. *Infect Immun* 69:5997–6003.
- Olafsdottir TA, Lingnau K, Nagy E, Jonsdottir I. (2012). Novel protein-based pneumococcal vaccines administered with the Th1-promoting adjuvant IC31 induce protective immunity against pneumococcal disease in neonatal mice. *Infect Immun* 80:461–8.
- Oliveira ML, Areas AP, Campos IB, et al. (2006). Induction of systemic and mucosal immune response and decrease in *Streptococcus pneumoniae* colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A. *Microbes Infect* 8:1016–24.
- Oliveira ML, Miyaji EN, Ferreira DM, et al. (2010). Combination of pneumococcal surface protein A (PspA) with whole cell pertussis vaccine increases protection against pneumococcal challenge in mice. *PLoS One* 5:e10863.
- Overweg K, Pericone CD, Verhoef GG, et al. (2000). Differential protein expression in phenotypic variants of *Streptococcus pneumoniae*. *Infect Immun* 68:4604–10.
- Paterson GK, Mitchell TJ. (2006). The role of *Streptococcus pneumoniae* sortase A in colonisation and pathogenesis. *Microbes Infect* 8:145–53.
- Paton JC. (1996). The contribution of pneumolysin to the pathogenicity of *Streptococcus pneumoniae*. *Trends Microbiol* 4:103–6.
- Paton JC. (2011). Vaccines against *Streptococcus pneumoniae*. In: Rappuoli R, Bagnoli F, ed. *Vaccine Design - Innovative approaches and Novel Strategies*. Caister Academic Press: Norfolk, UK, 303–28.
- Paton JC, Lock RA, Lee CJ, et al. (1991). Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. *Infect Immun* 59:2297–304.
- Perciani CT, Barazzone GC, Goulart C, et al. (2013). Conjugation of Polysaccharide 6B from *Streptococcus pneumoniae* with Pneumococcal Surface Protein A: PspA conformation and its effect on the immune response. *Clin Vaccine Immunol* 6:858–66.
- Pimenta FC, Miyaji EN, Areas AP, et al. (2006). Intranasal immunization with the cholera toxin B subunit-pneumococcal surface antigen A fusion protein induces protection against colonization with *Streptococcus pneumoniae* and has negligible impact on the nasopharyngeal and oral microbiota of mice. *Infect Immun* 74:4939–44.
- Polissi A, Pontiggia A, Feger G, et al. (1998). Large-scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect Immun* 66:5620–9.
- Rapola S, Jantti V, Haikala R, et al. (2000). Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *J Infect Dis* 182:1146–52.
- Ren B, Li J, Genschmer K, et al. (2012). The absence of PspA or presence of antibody to PspA facilitates the complement-dependent phagocytosis of pneumococci *in vitro*. *Clin Vaccine Immunol* 19:1574–82.

- Rioux S, Neyt C, DI Paolo E, et al. (2011). Transcriptional regulation, occurrence and putative role of the PhT family of *Streptococcus pneumoniae*. *Microbiology* 157:336–48.
- Roche H, Hakansson A, Hollingshead SK, Briles DE. (2003). Regions of PspA/EF3296 best able to elicit protection against *Streptococcus pneumoniae* in a murine infection model. *Infect Immun* 71:1033–41.
- Salha D, Szeto J, Myers L, et al. (2012). Neutralizing antibodies elicited by a novel detoxified pneumolysin derivative, PlyD1, provide protection against both pneumococcal infection and lung injury. *Infect Immun* 80:2212–20.
- Sanchez-Beato AR, Lopez R, Garcia JL. (1998). Molecular characterization of PcpA: a novel choline-binding protein of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* 164:207–14.
- Santamaria R, Goulart C, Perciani CT, et al. (2011). Humoral immune response of a pneumococcal conjugate vaccine: capsular polysaccharide serotype 14-Lysine modified PspA. *Vaccine* 29:8689–95.
- Santolaya ME, O'ryan ML, Valenzuela MT, et al. (2012). Immunogenicity and tolerability of a multicomponent meningococcal serogroup B (4CMenB) vaccine in healthy adolescents in Chile: a phase 2b/3 randomised, observer-blind, placebo-controlled study. *Lancet* 379:617–24.
- Saskova L, Novakova L, Basler M, Branny P. (2007). Eukaryotic-type serine/threonine protein kinase StkP is a global regulator of gene expression in *Streptococcus pneumoniae*. *J Bacteriol* 189:4168–79.
- Seepersaud R, Hanniffy SB, Mayne P, et al. (2005). Characterization of a novel leucine-rich repeat protein antigen from group B streptococci that elicits protective immunity. *Infect Immun* 73:1671–83.
- Seiberling M, Bologna M, Brookes R, et al. (2012). Safety and immunogenicity of a pneumococcal histidine triad protein D vaccine candidate in adults. *Vaccine* 30:7455–60.
- Selva L, Ciruela P, Blanchette K, et al. (2012). Prevalence and clonal distribution of PcpA, PsrP and Pilus-I among pediatric isolates of *Streptococcus pneumoniae*. *PLoS One* 7:e41587.
- Shah P, Marquart M, Quin LR, Swiatlo E. (2006). Cellular location of polyamine transport protein PotD in *Streptococcus pneumoniae*. *FEMS Microbiol Lett* 261:235–7.
- Shah P, Romero DG, Swiatlo E. (2008). Role of polyamine transport in *Streptococcus pneumoniae* response to physiological stress and murine septicemia. *Microbial Pathogen* 45:167–72.
- Shah P, Swiatlo E. (2006). Immunization with polyamine transport protein PotD protects mice against systemic infection with *Streptococcus pneumoniae*. *Infect Immun* 74:5888–92.
- Shaper M, Hollingshead SK, Benjamin JR WH, Briles DE. (2004). PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. *Infect Immun* 72:5031–40.
- Shi H, Wang S, Roland KL, et al. (2010). Immunogenicity of a live recombinant *Salmonella enterica* serovar typhimurium vaccine expressing *pspA* in neonates and infant mice born from naive and immunized mothers. *Clin Vaccine Immunol* 17:363–71.
- Spraggon G, Koesema E, Scarselli M, et al. (2010). Supramolecular organization of the repetitive backbone unit of the *Streptococcus pneumoniae* pilus. *PLoS One* 5:e10919.
- Talkington DF, Brown BG, Tharpe JA, et al. (1996). Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). *Microb Pathog* 21:17–22.
- Tettelin H, Nelson KE, Paulsen IT, et al. (2001). Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 293:498–506.
- Tong HH, Li D, Chen S, et al. (2005). Immunization with recombinant *Streptococcus pneumoniae* neuraminidase NanA protects chinchillas against nasopharyngeal colonization. *Infect Immun* 73:7775–8.
- Tseng HJ, McEwan AG, Paton JC, Jennings MP. (2002). Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. *Infect Immun* 70:1635–9.
- Vela Coral MC, Fonseca N, Castaneda E, et al. (2001). Pneumococcal surface protein A of invasive *Streptococcus pneumoniae* isolates from Colombian children. *Emerg Infect Dis* 7:832–6.
- Vintini E, Villena J, Alvarez S, Medina M. (2010). Administration of a probiotic associated with nasal vaccination with inactivated *Lactococcus lactis*-PspA induces effective protection against pneumococcal infection in young mice. *Clin Exp Immunol* 159:351–62.
- Virolainen A, Russell W, Crain MJ, et al. (2000). Human antibodies to pneumococcal surface protein A in health and disease. *Pediatr Infect Dis J* 19:134–8.
- Wang S, Li Y, Shi H, et al. (2010). Immune responses to recombinant pneumococcal PsaA antigen delivered by a live attenuated *Salmonella* vaccine. *Infect Immun* 78:3258–71.
- Ware D, Watt J, Swiatlo E. (2005). Utilization of putrescine by *Streptococcus pneumoniae* during growth in choline-limited medium. *J Microbiol* 43:398–405.
- Weinberger DM, Malley R, Lipsitch M. (2011). Serotype replacement in disease after pneumococcal vaccination. *Lancet* 378:1962–73.
- Whalan RH, Funnell SG, Bowler LD, et al. (2005). PiuA and Piao, iron uptake lipoproteins of *Streptococcus pneumoniae*, elicit serotype independent antibody responses following human pneumococcal septicaemia. *FEMS Immunol Med Microbiol* 43:73–80.
- WHO. (2006). State of the art of new vaccine and development. Immunization, Vaccines and Biologicals Available from: whqlibdoc.who.int/hq/2006/who_ivb_06.01_eng.pdf [Last accessed 6 May 2013].
- Weiser NJ. (2010). The pneumococcus: why a commensal misbehaves. *J Mol Med* 88:97–102.
- Whaley MJ, Sampson JS, Johnson SE, et al. (2010). Concomitant administration of recombinant PsaA and PCV7 reduces *Streptococcus pneumoniae* serotype 19A colonization in a murine model. *Vaccine* 28:3071–5.
- Witzenrath M, Pache F, Lorenz D, et al. (2011). The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol* 187:434–40.
- Wizemann TM, Heinrichs JH, Adamou JE, et al. (2001). Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. *Infect Immun* 69:1593–8.
- Wright AK, Ferreira DM, Gritzfeld JF, et al. (2012). Human nasal challenge with *Streptococcus pneumoniae* is immunising in the absence of carriage. *PLoS pathogens* 8:e1002622.
- Wu K, Zhang X, Shi J, et al. (2010). Immunization with a combination of three pneumococcal proteins confers additive and broad protection against *Streptococcus pneumoniae* Infections in Mice. *Infect Immun* 78:1276–83.
- Yuan ZQ, Lv ZY, Gan HQ, et al. (2011). Intranasal immunization with autolysin (LytA) in mice model induced protection against five prevalent *Streptococcus pneumoniae* serotypes in China. *Immunol Res* 51:108–15.
- Zhang Y, Masi AW, Barniak V, et al. (2001). Recombinant PspA protein, a unique histidine motif-containing protein from *Streptococcus pneumoniae*, protects mice against intranasal pneumococcal challenge. *Infect Immun* 69:3827–36.
- Zhong W, Xu W, Wang H, et al. (2012). Mucosal immunization with caseinolytic protease X elicited cross-protective immunity against pneumococcal infection in mice. *Exp Biol Med* (Maywood) 237:694–702.

Analysis of the coverage capacity of the StreptInCor candidate vaccine against *Streptococcus pyogenes*

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ABSTRACT

Streptococcus pyogenes is responsible for infections as pharyngitis, sepsis, necrotizing fasciitis and streptococcal toxic shock syndrome. The M protein is the major bacterial antigen and consists of both polymorphic N-terminal portion and a conserved region. In the present study, we analyzed the *in vitro* ability of StreptInCor a C-terminal candidate vaccine against *S. pyogenes* to induce antibodies to neutralize/opsonize the most common *S. pyogenes* strains in Sao Paulo by examining the recognition by sera from StreptInCor immunized mice. We also evaluated the presence of cross-reactive antibodies against human heart valve tissue. Anti-StreptInCor antibodies were able to neutralize/opsonize at least 5 strains, showing that immunization with StreptInCor is effective against several *S. pyogenes* strains and can prevent infection and subsequent sequelae without causing autoimmune reactions.

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1. Introduction

Streptococcus pyogenes causes diseases as pharyngitis, impetigo, streptococcal toxic shock syndrome and necrotizing fasciitis. Rheumatic fever (RF), acute streptococcal glomerulonephritis and rheumatic heart disease (RHD) are non-suppurative autoimmune post-streptococcal sequelae that arise from a delayed immune response to infection in genetically predisposed individuals [1]. Several markers are described as risk factors for RF/RHD, including HLA-DR7, the allele most commonly associated with RHD in Brazil and other countries [2].

According to the World Health Organization (WHO), *S. pyogenes* is responsible for 15–20% of bacterial pharyngitis cases, which primarily affect 5- to 18-year-old individuals [3]. The incidence of bacterial pharyngitis varies among countries, and even within the same country, there are variations in different regions due to age, socioeconomic and environmental factors and quality of health services [4,5].

The M protein has been described as the major bacterial antigen [6]. The protein consists of two polypeptide chains in an alpha double helix coiled-coil that forms fibrils extending up to 60 nm away from the bacterial surface. It is approximately 450 amino acids long and is divided into tandem repeat blocks distributed over four regions (A, B, C and D). The N-terminal portion (regions A and B) is polymorphic and differences within the first 150 amino acid residues of the A region allow for the classification of different serotypes [7,8]. The C-terminal portion (regions C and D) is highly conserved, responsible for binding the bacteria to the oropharynx mucosa and has antiphagocytic properties [6,7].

RF/RHD pathogenesis is related to the production of autoantibodies and autoreactive T cells that recognize and cross-react with epitopes from both the M protein and human heart tissue by molecular mimicry [9,10] and it was demonstrated by analyzing the T cell repertoire that infiltrated cardiac tissue and led to damage in RHD [11].

M1 is the most common strain worldwide and, due to its high virulence, is involved in invasive and non-invasive infections in several countries [12,13]. There is a large diversity of strains in Brazil. The most prevalent strains found in a sample from Sao Paulo city were the M1, M6, M12, M22, M77 and M87 compatible with those found in the rich districts from Salvador [5,14]. These M-types are also predominant in most of the world western countries [15].

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Besides that, there is a much higher diversity of M-types in the poor districts from Salvador and Brasilia typically found in low incomes regions [5,16].

The classification of strains according to their tissue tropism for throat (A–C pattern), skin (D pattern) or both (E pattern) is based on the organization of *emm* and *emm*-like genes located in the *mga* locus within *S. pyogenes* genome and constitute the base for *emm* pattern genotyping [17,18]. Strains belong to A–C pattern, as the M1, M6 and M12, has been historically associated with rheumatic fever [10,19] while the M22 and M87 strains belong to the E pattern are considered a group associated with both throat and skin infections [19,20].

The development of a vaccine against *S. pyogenes* would provide many benefits, preventing streptococcal infections and sequelae. Several vaccine development studies have focused on the M protein due to its high immunogenicity and have been tested since 1923 [21,22]. The first vaccines used whole inactivated bacteria. The use of the entire M protein from specific strains started in 1979, but the results were not satisfactory. In the 1980s, synthetic peptide models were introduced. Later, molecular biology models based on the N-terminal portion were developed, and hexavalent and 26-valent vaccines containing the most prevalent serotypes in United States entered into phase I/II clinical trials [23]. Simultaneously, new approaches for defining protective epitopes were designed based on both N and C-terminal regions. Currently, researchers are studying models that are based on streptococcal antigens other than the M protein [24].

Approximately 15 years ago, our group started to develop an effective vaccine against *S. pyogenes*. The approach considered how the immune system could be more effective in inducing a protective immune response via T and B lymphocytes without triggering autoimmunity [25].

Briefly, the vaccine is based on amino acid sequences from the M5 protein conserved region (C2 and C3 regions). Reactivity was evaluated by humoral and cellular analyses to define potentially protective epitopes. The B epitope, composed of 22 amino acid residues, is linked by 8 amino acid residues to the T epitope, which consists of 25 amino acid residues, using a segment of the natural M5 protein. We synthesized a peptide with 55 residues called StreptInCor (medical ID), which contained both the B and T epitopes [25].

The analysis of StreptInCor sequence binding to different HLA class II molecules was conducted using theoretical possibilities of processed peptides to fit into the pockets of antigen presenting cells (APC), followed by T cell activation via T cell receptor (TCR) that stimulates B cells to secrete antibodies with protective potential. The StreptInCor sequence contain seven potential binding sites that were recognized by HLA class II (DRB1*/DRB3*/DRB4*/DRB5*), making StreptInCor a candidate vaccine with broad capacity of coverage [26].

The vaccine peptide was tested in animal models. Inbred and outbred mice showed strong humoral response against StreptInCor with high IgG production [27]. Challenge with M1 strain in immunized Swiss mice showed a survival rate of 100% for up to 21 days, compared to the control group's lower survival rate (40%) [28]. HLA class II transgenic mice, that have the capacity of present the vaccine epitope to the TCR in the context of human molecules, were immunized with StreptInCor in aluminum hydroxide (Alum) and produced high titers of IgG1 (Th2-dependent IL-4) and IgG2a (Th1-dependent IFN- γ). Specific antibodies were observed after a period of one year without reactivity against human heart proteins. No lesions were observed in several organs [29], indicating that StreptInCor is safe and has protection potential.

In the present study, we analyzed the *in vitro* ability of anti-StreptInCor antibodies to neutralize/opsonize *S. pyogenes* strains frequently found in Sao Paulo. We also analyzed the absence of

humoral autoimmune reactions against human heart valve tissue.

The results presented here showed that anti-StreptInCor antibodies were able to neutralize/opsonize M1, M5, M12, M22 and M87 *S. pyogenes* strains, indicating that the vaccine can be effective against the bacteria, preventing infection and subsequent sequelae without causing autoimmune reactions.

2. Methods

2.1. StreptInCor vaccine epitope

The vaccine epitope consists of the following 55 amino acid residues: KGLRRDLASREAKKQLEAEQQKLEQNKISEASRKGLRRDLASREAKKQVEKA. The peptide was synthesized using a 9- α -fluorenylmethoxy-carbonyl (Fmoc) solid-phase strategy, purified by reverse phase high-pressure liquid chromatography (RP-HPLC, Shimadzu, Japan). Peptide quality was assessed by matrix-assisted desorption ionization mass spectrometry (MALDI-ToF, Ettan Maldi ToF Pro, Amersham-Pharmacia, Sweden) as previously described [25]. Patents PCT-BR07/000184.

2.2. Mice

Inbred BALB/c and outbred Swiss mice with mature immune system (6- to 8-week-old) specific pathogen-free from CEMIB (Unicamp, Campinas, Brazil) were maintained in autoclaved cages (Alesco, Brazil) and handled under sterile conditions in the animal facility at the Tropical Medicine Institute, University of São Paulo, Brazil. Procedures were performed in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines approved by the Tropical Medicine Institute Ethics Committee (project number 002/08).

2.3. Immunization

Mice sera previously immunized with 10 μ g of StreptInCor adsorbed onto 60 μ g of aluminum hydroxide gel (Sigma–Aldrich Corp., USA) in saline via subcutaneous with two doses 14 days apart. Animals that received saline plus 60 μ g of adjuvant were used as negative controls. Positive controls were immunized with recombinant streptococcal M1 full protein (clone kindly provided by Prof. Patrick Cleary, University of Minnesota Medical School, MN, USA), produced and purified in our lab. Sera samples were obtained under light anesthesia by retro-orbital puncture on day 28 following immunization. Samples with high specific antibody titers (>1:1.200) detected by Enzyme-Linked Assay Immunoabsorbent (ELISA) [28] were used.

2.4. *S. pyogenes* strains

The strains were obtained from patients treated at the Clinical Hospital, University of Medicine – Sao Paulo, between 2001 and 2008 and identified by genotyping [30]. The M1, M5, M6, M12, M22 and M87 specimens were cultured on sheep blood agar (Vetec, Brazil), followed by growth in Todd-Hewitt broth (Himedia, India) until OD₆₀₀ of 0.4 and stored at -80°C .

2.5. M protein C-terminal region sequence alignment of different *S. pyogenes* strains

Amino acid sequences from the M protein C-terminal region of M1, M5, M6, M12 and M87 strains were aligned using the StreptInCor amino acid sequence through the online program BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences are available at Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed>),

Swissprot (<http://www.uniprot.org/help/uniprotkb>) and CDC (<http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm>). The alignment was colored using the Jalview 2.7 program with Zap0 staining to indicate the amino acids' chemical groups.

2.6. Neutralization assay by flow cytometry

S. pyogenes isolates were cultured as described in Section 2.4. The bacteria were incubated with 1:100 BALB/c hyperimmune or control mice sera ($n=9$) for 30 min. After, samples were incubated with murine IgG phycoerythrin (PE) – (Invitrogen, USA) specific antibody (1:50) for 30 min. After, washed and fixed in 1% paraformaldehyde. Subsequently, 10,000 events were acquired using a flow cytometer FACS Canto II (BD Biosciences, USA), and the results were analyzed using FlowJo software version 3.4.1. Statistical analysis was performed using Mann–Whitney test after analyzing normalization using the Shapiro–Wilk test.

2.7. *S. pyogenes* strains Western Blotting

M1 and M5 strains were cultured as described in Section 2.4. The bacteria were disrupted by sonication (Sonic Dismembrator 60, Thermo Fisher Scientific, Sweden). The proteins were precipitated in TCA/Acetone solution at -20°C and concentrated in filter columns (Millipore, USA). The Bradford assay (Bradford, 1976) was used for quantitation of proteins (Bio-Rad, USA). After SDS–PAGE electrophoresis, the gel was blotted onto nitrocellulose membranes [31,32], subsequently blocked with Tris-buffered saline containing 5% skim milk. The membrane was treated with immunized or control BALB/c mice sera pools ($n=6$), incubated with anti-mouse IgG alkaline phosphatase and revealed with NBT-BCIP solution (Invitrogen, USA). The molecular weight marker used was Full-range Rainbow (GE Healthcare, Sweden). Membranes and gels images were obtained using an ImageScanner photo-scanner with the scanning software Labscan (GE Healthcare, Sweden). Densitometry was performed by TL ImageQuant software (GE Healthcare, Sweden).

2.8. Opsonophagocytic assay

S. pyogenes strains were cultured until they reached an optical density of 0.4–0.5. After, approximately 2.5×10^6 colony-forming units (CFU) were incubated with 1:100 anti-StreptInCor or control sera ($n=6$) from BALB/c mice, previously heat-inactivated by incubation at 56°C for 30 min, to destroy the activity of serum complement. Pre-immunization sera from 6 BALB/c mice were used as negative control. After incubation, 10% of normal mouse serum (NMS) was added as complement source. To stimulate the recruitment of mice immune cells, $10\text{ }\mu\text{g}$ of Concanavalin A (Canavalia ensiformis-ConA, Sigma) was injected intraperitoneally. The animals were sacrificed 48 h after injection, and the peritoneal cavity was washed with 5 mL of cold PBS on ice. The concentration of peritoneal cells was adjusted to $4 \times 10^6/\text{mL}$ in HBSS (Invitrogen, USA) containing 0.01% gelatin (opsonization buffer). The bacteria treated with hyperimmune or control mice sera were harvested and incubated with 4×10^5 peritoneal cells at 37°C for 45 min with shaking (220 rpm). Ten-fold dilutions of the samples were performed and $10\text{ }\mu\text{L}$ aliquots of each dilution were cultured on blood agar plates. The count live colonies were performed as previously described [33]. After 20 min, slides of the M1 strain opsonophagocytic assay were prepared by cytospin, stained with Instant-Prov (Newprov, Brazil), subsequently analyzed by light microscopy using an Axion Vision Zeiss Imager A1 and photographed by Axion Vision software (Zeiss, Germany). Statistical analysis was performed using Kruskal–Wallis test.

2.9. Heart tissue valve Western Blotting

Heart tissue was obtained from the lysate of a postmortem normal human mitral valve, separated by SDS–PAGE and blotted onto nitrocellulose membranes [31,32]. The blots were blocked with Tris-buffered saline containing 5% skim milk. The membrane was sequentially treated with a pool ($n=6$) of BALB/c or Swiss immunized mice sera and anti-mouse IgG alkaline phosphatase and revealed with NBT-BCIP solution (Invitrogen, USA).

3. Results

3.1. Anti-StreptInCor recognized M1 and M5 strains

We observed that anti-StreptInCor antibodies from the BALB/c mice sera pool were able to cross-recognize both the M5 and M1 proteins in total protein extracts from each strain (Fig. 1).

3.2. Several streptococcal strains were neutralized by anti-StreptInCor antibodies

The anti-StreptInCor antibodies from Swiss mice were able to neutralize the M1, M5, M12, M22 and M87 strains by cross-recognizing the M protein on the bacterial surface with a Median Fluorescence Intensity (MFI) 2 or 3 times greater than the MFI of control sera (Fig. 2).

3.3. Anti-StreptInCor antibodies were able to opsonize, and promote the phagocytosis and death of several strains

Anti-StreptInCor antibodies from BALB/c and Swiss mice were able to promote opsonophagocytosis and death of the M1, M5, M12, M22 and M87 strains (Fig. 3a and b, respectively). The amino acid sequences alignment of the M protein C-terminal region of the strains used in this study had, on average, 72% identity with the StreptInCor amino acid sequence (Fig. 3c). The M1, M6 and M12 strains had an additional block of 7 amino acids, while the M87 strain contained two fewer amino acids than the StreptInCor sequence. M1 strain was killed in peritoneal cells by phagocytosis 20 min after the opsonization assay as observed by optical microscopy (Fig. 4a–d).

3.4. StreptInCor did not induce autoimmune reactions

No autoreactive antibodies against human heart mitral valve protein extracts were observed (Fig. 5).

4. Discussion

The development of a vaccine against multiple *S. pyogenes* strains without causing autoimmunity will bring numerous benefits to human health. A vaccine would prevent streptococcal infections and sequelae and could be more effective and longer-lasting than the currently used treatment.

In addition to have broad coverage against strains, a vaccine should promote the production of neutralizing and opsonophagocytic antibodies, which are the body's major defense lines against extracellular microorganisms.

In the 70 and 80s several models of anti *S. pyogenes* vaccines were assayed without satisfactory results, however by using new approaches several models were proposed [24]. Strain-specific vaccines based on recombinant N-terminal portions of the M protein serotypes most prevalent in the US entered into phase I/II clinical trials [23]. A new approach based on the 30 most prevalent serotypes is being tested and the results indicate that the vaccine

Fig. 1. Reactivity of sera pool from BALB/c mice immunized with StreptInCor and controls against M1 and M5 protein extracts. Western blotting of (a), M1 protein extract (b), M5 protein extract; lanes: (1) molecular weight marker, (2) anti-recombinant M1 sera pool, (3) pre-immune sera pool, (4) control sera pool, (5) anti-StreptInCor sera pool. M1 and M5 molecular weights are available at <http://www.uniprot.org/help/uniprotkb>. The pool was composed of 6 BALB/c mice sera per group.

could evoke cross-protective antibodies capable of covering most of the serotypes not included in the vaccine design [34]. Therefore, as the prevalence of strains can vary depending on the region of the world a vaccine based on the conserved region of the M protein probably will present a broad coverage.

The StreptInCor is a vaccine model developed from the M5 protein C-terminal region [25], specifically located on C2 and C3 region that is conserved among the serotypes. It is interesting to note that the sequences KLEEQNKI that link both the T and B epitopes in the StreptInCor peptide, is located after the C0–C1, C1–C2, C2–C3 conserved linkers as showed by McMillan et al. (2013) [19], and this sequence is in accordance with the natural M5 protein segment.

Antibodies induced by the vaccination should be capable of binding to the same cross-conserved region of the M protein from different *S. pyogenes* strains around the world. This process would neutralize the adhesion function, leading to phagocytosis and killing by APCs.

We observed that immunization with StreptInCor in mice was able to promote the antibody production against C-terminal

epitopes capable of cross-recognizing similar regions in both the M5 and M1 proteins. In addition, anti-StreptInCor neutralizing antibodies had the capacity to bind to M1, M5, M12, M22 and M87 proteins on the surface of each bacterial cell, opsonizing and leading to phagocytosis and death as observed in the opsonophagocytic assays. The M1 strain, the most common worldwide, also one of the most virulent strains [12], was rapidly killed on the APCs phagocytosis vacuoles induced by StreptInCor immunization, as compared with controls. These results indicate the capacity of anti-StreptInCor antibodies to neutralize/opsonize the most prevalent strains.

By amino acid sequences alignment in the present study, we observed that the C-terminal region of the M proteins had, on average, 72% identity with StreptInCor. The M1, M6 and M12 have an additional block of 7 amino acid residues in their sequences, while M87 has two fewer amino acids than the StreptInCor sequence. These differences did not interfere with antibody recognition, as observed in the opsonization assays with several strains.

Fig. 2. Neutralization of different strains by anti-StreptInCor antibodies. Analysis of neutralization of M1, M5, M12, M22 and M87 strains was performed by flow cytometry; the Median Fluorescence Intensity (MFI) obtained for each serum sample is represented as data point. Anti-StreptInCor BALB/c mice sera ($\blacktriangle$); control BALB/c mice sera ($\bullet$); *P value < 0.5; **P value < 0.1; ***P value < 0.01; ****P value < 0.001 (Mann–Whitney). Data are representative of three independent experiments with nine mice per group. The error bar represents the standard deviation (SD).

Fig. 3. Opsonophagocytosis and death of several *S. pyogenes* strains by mice peritoneal cells. Opsonophagocytosis of M1, M5, M12, M22 and M87 strains by anti-StreptInCor antibodies from: (a) BALB/c mice sera ($n=6$), (b) Swiss mice sera ($n=6$), immunized with $10\text{ }\mu\text{g}$ of StreptInCor or controls. The CFU units obtained at 10^6 dilution are represented as a data point. Anti-StreptInCor BALB/c mice sera ($\blacktriangle$); control BALB/c mice sera ($\bullet$); pre-immune mice sera pool ($\diamond$). * P value <0.05 ; ** P value <0.01 ; *** P value <0.001 ; **** P value <0.0001 (Kruskal–Wallis). Data are representative of three independent experiments with six mice per group. The error bar represents the standard deviation (SD). (c) C-terminal amino acid sequences from M1, M5, M6, M12 and M87 strains aligned with the StreptInCor amino acid sequence; identity percentage is represented in red; (*) identical amino acid residues; (----): lack of residues; (°) different amino acids from the same chemical group. Colors – blue and red: amino acids with electrically charged side chains; green: amino acids with uncharged polar side chains; pink: glycines; light orange: amino acids with hydrophobic side chains. Different colors in the same column indicate an amino acid from a different chemical group; (—) natural link between the B and T cell epitopes.

Fig. 4. Opsonization, phagocytosis and death of M1 strain induced by anti-StreptInCor antibodies. Optical microscopy of M1 strain phagocytosis and death within peritoneal cells stained with Instant-Prov. Acquisition at ocular lenses 20 min after the opsonization assay. Magnification $10\times$, objective ranged from $20\times$ to $40\times$. Bacteria treated with pre-immune control sera (a) magnification of $200\times$, (b) magnification of $400\times$; bacteria treated with hyperimmune sera (c), magnification of $200\times$; (d) magnification of $400\times$. Black arrows: *S. pyogenes*; red arrows: *S. pyogenes* phagocytosed by APC; blue arrows: *S. pyogenes* in APC digestive vacuoles.

Fig. 5. Autoimmune reactivity evaluation. No autoimmune antibodies against human heart valve protein extract were observed by Western Blotting. (a) SDS-Page post-transfer, (b) Western blotting membrane; lanes: (1) molecular weight marker; (2) anti-human myosin antibody; (3) isotype control; (4) pre-immune mice sera pool; (5) control BALB/c mice sera pool; (6) anti-StreptInCor BALB/c mice sera pool; (7) control Swiss mice sera pool; (8) anti-StreptInCor Swiss mice sera pool. The pool was composed of 6 mice sera per group.

In addition, M-types amino acid sequences from UniprotKB database were aligned in the Short-Blastp program against StreptInCor. The results showed that the StreptInCor sequence is on average 71% conserved amongst the 541M protein sequences available at the public database. This block of results indicates that anti-StreptInCor antibodies can bind directly to multiple parts of the M protein C-terminal sequences due to the repeat blocks of amino acids. Consequently, differences between StreptInCor and the M protein sequences do not affect opsonization of the target strain, indicating that StreptInCor have broad capacity of coverage against the diverse M-types around the world.

Previously we showed that StreptInCor can be recognized by several HLA class II molecules, making it a candidate vaccine with broad capacity of coverage. The binding prediction of the C-terminal amino acid sequences of the M1, M5, M6, M12 and M87 proteins with different HLA class II molecules shows that the possibility of recognition/processing of M proteins and peptides in the pockets (P1, P4, P6 and P9) of different HLA class II molecules agree with previous human studies from our group [26].

Another important data present here is that the anti-StreptInCor opsonizing and neutralizing antibodies did not induce cross-reactivity with human valve protein extracts, indicating the absence of cross-reactive antibodies. These results agrees with previous studies with HLA class II transgenic mice, in which no cross reactivity against heart-tissue derived proteins and no tissue lesions were observed in several organs up to one year post-vaccination [29].

5. Conclusions

The present work reinforces the safety of and strong immune response triggered by the StreptInCor mice vaccination. Productions of antibodies that opsonize and neutralize a broad range of *S. pyogenes* strains indicate the potential of StreptInCor to prevent streptococcal infections without causing deleterious reactions.

Conflict of interest

The authors declare that there is no conflict of interest.

Intellectual properties

StreptInCor intellectual properties are in the names of Luiza Guilherme and Jorge Kalil.

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References

- [1] Narula J, Virmani R, Reddy KS, Tandon R. Rheumatic fever. American Registry of Pathology: Washington, DC; 1999.
- [2] Guilherme L, Köhler KF, Postol E, Genes KJ. Autoimmunity and pathogenesis of rheumatic heart disease. *Ann Pediatr Cardiol* 2011;4:13–21.
- [3] Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. *Lancet Infect Dis* 2005;5:685–94.
- [4] Barbosa PJB, Müller RE, Latado AL, Achutti AC, Ramos AIO, Weksler C, et al. Diretrizes Brasileiras para Diagnóstico, Tratamento e Prevenção da Febre Reumática da Sociedade Brasileira de Cardiologia, da Sociedade Brasileira de Pediatria e da Sociedade Brasileira de Reumatologia. *Arquivos Brasileiros de Cardiologia* 2009;93(3 Suppl. 4):1–18.
- [5] Tartof SY, Reis JN, Andrade AN, Ramos RT, Reis MG, Riley LW. Factors associated with Group A streptococcus emm type diversification in a large urban setting in Brazil: a cross-sectional study. *BMC Infect Dis* 2010;10:327.
- [6] Smeesters PR, McMillan DJ, Sriprakash KS. The streptococcal M protein: a highly versatile molecule. *Trends Microbiol* 2010;18:275–82.
- [7] Bisno AL, Brito MO, Collins CM. Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* 2003;3:191–200.
- [8] McMillan DJ, Drèze P-A, Vu T, Bessen DE, Guglielmini J, Steer AC, et al. Updated model of group A *Streptococcus* M proteins based on a comprehensive worldwide study. *Authors Clin Microbiol Infect* 2012;5:222–9.
- [9] Guilherme L, Kalil J, Cunningham M. Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. *Autoimmunity* 2006;39:31–9.
- [10] Cunningham MW. Pathogenesis of group A streptococcal infections. *Clin Microbiol Rev* 2000;13:470–511.
- [11] Guilherme L, Cunha-Neto E, Coelho V, Snitcowsky R, Pomerantzeff PM, Assis RV, et al. Human heart-infiltrating T-cell clones from rheumatic heart disease patients recognize both streptococcal and cardiac proteins. *Circulation* 1995;92:415–20.
- [12] Steer AC, Law I, Matatolu L, Beall BW, Carapetis JR. Global emm type distribution of group A streptococci: systematic review and implications for vaccine development. *Lancet Infect Dis* 2009;9:611–6.

- [13] Smeesters PR, McMillan DJ, Sriprakash KS, Georgousakis MM. Differences among group A streptococcus epidemiological landscapes: consequences for M protein-based vaccines. *Expert Rev Vaccines* 2009;8:1705–20.
- [14] Guilherme L, Freschi de Barros S, Tanaka AC, Ribeiro Castro M, Kalil J. *S. Pyogenes* infections and its sequelae. In: Prabhakar SS, editor. *An update on Glomerulopathies – Clinical and Treatment Aspects*. InTech; 2011.
- [15] Shulman ST, Tanz RR, Dale JB, Beall B, Kabat W, Kabat K, et al. Seven-year surveillance of North American pediatric group A streptococcal pharyngitis isolates. *Clin Infect Dis* 2009;49:78–84.
- [16] Smeesters PR, Vergison A, Campos D, de Aguiar E, Miendje Deyi VY, van Melder L. Differences between Belgian and Brazilian group A *Streptococcus* epidemiologic landscape. *PLoS One* 2006;1:e10.
- [17] McGregor KF, Spratt BG, Kalia A, Bennett A, Bilek N, Beall B, et al. Multilocus sequence typing of *Streptococcus pyogenes* representing most known emm types and distinctions among subpopulation genetic structures. *J Bacteriol* 2004;186:4285–94.
- [18] Bessen DE, Carapetis JR, Beall B, Katz R, Hibble M, Currie BJ, et al. Contrasting molecular epidemiology of group A streptococci causing tropical and nontropical infections of the skin and throat. *J Infect Dis* 2000;182:1109–16.
- [19] McMillan DJ, Drèze PA, Vu T, Bessen DE, Guglielmini J, Steer AC, et al. Updated model of group A *Streptococcus* M proteins based on a comprehensive world-wide study. *Clin Microbiol Infect* 2013;19:E222–9.
- [20] Smeesters PR, Mardulyn P, Vergison A, Leplae R, van Melder L. Genetic diversity of Group A *Streptococcus* M protein: implications for typing and vaccine development. *Vaccine* 2008;26:5835–42.
- [21] Steer AC, Dale JB, Carapetis JR. Progress toward a global group A streptococcal vaccine. *Pediatr Infect Dis J* 2013;32:180–2.
- [22] Dale JB, Fischetti VA, Carapetis JR, Steer AC, Sow S, Kumar R, et al. Group A streptococcal vaccines: paving a path for accelerated development. *Vaccine* 2013;31(Suppl. 2):B216–22.
- [23] McNeil SA, Halperin SA, Langley JM, Smith B, Warren A, Sharratt GP, et al. Safety and immunogenicity of 26-valent group A streptococcus vaccine in healthy adult volunteers. *Clin Infect Dis* 2005;41:1114–22.
- [24] Steer AC, Batzloff MR, Mulholland K, Carapetis JR. Group A streptococcal vaccines: facts versus fantasy. *Curr Opin Infect Dis* 2009;22:544–52.
- [25] Guilherme L, Faé KC, Higa F, Chaves L, Oshiro SE, Freschi de Barros S, et al. Towards a vaccine against rheumatic fever. *Clin Dev Immunol* 2006;13:125–32.
- [26] Guilherme L, Alba MP, Ferreira FM, Oshiro SE, Higa F, Patarroyo ME, et al. Anti-group A streptococcal vaccine epitope: structure, stability, and its ability to interact with HLA class II molecules. *J Biol Chem* 2011;286:6989–98.
- [27] Guilherme L, Postol E, Freschi de Barros S, Higa F, Alencar R, Lastre M, et al. A vaccine against *S. pyogenes*: design and experimental immune response. *Methods* 2009;49:316–21.
- [28] Postol E, Alencar R, Higa FT, Freschi de Barros S, Demarchi LM, Kalil J, et al. StreptInCor: a candidate vaccine epitope against *S. pyogenes* infections induces protection in outbred mice. *PLoS One* 2013;8:e60969.
- [29] Guerino MT, Postol E, Demarchi LM, Martins CO, Mundel LR, Kalil J, et al. HLA class II transgenic mice develop a safe and long lasting immune response against StreptInCor, an anti-group A streptococcus vaccine candidate. *Vaccine* 2011;29(46):8250–6.
- [30] Guilherme L, Barros SFd, Tanaka AC, Castro MCR, Kalil J. *S. pyogenes* infection and its sequelae. *Croácia: Rijeka*; 2011. p. 468.
- [31] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.
- [32] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4.
- [33] Goulart C, Darrieux M, Rodriguez D, Pimenta FC, Brandileone MC, de Andrade AL, et al. Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. *Vaccine* 2011;29:1634–42.
- [34] Dale JB, Penfound TA, Tamboura B, Sow SO, Nataro JP, Tapia M, et al. Potential coverage of a multivalent M protein-based group A streptococcal vaccine. *Vaccine* 2013;31:1576–81.

Conjugation of Polysaccharide 6B from *Streptococcus pneumoniae* with Pneumococcal Surface Protein A: PspA Conformation and Its Effect on the Immune Response

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Despite the substantial beneficial effects of incorporating the 7-valent pneumococcal conjugate vaccine (PCV7) into immunization programs, serotype replacement has been observed after its widespread use. As there are many serotypes currently documented, the use of a conjugate vaccine relying on protective pneumococcal proteins as active carriers is a promising alternative to expand PCV coverage. In this study, capsular polysaccharide serotype 6B (PS6B) and recombinant pneumococcal surface protein A (rPspA), a well-known protective antigen from *Streptococcus pneumoniae*, were covalently attached by two conjugation methods. The conjugation methodology developed by our laboratory, employing 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as an activating agent through carboxamide formation, was compared with reductive amination, a classical methodology. DMT-MM-mediated conjugation was shown to be more efficient in coupling PS6B to rPspA clade 1 (rPspA1): 55.0% of PS6B was in the conjugate fraction, whereas 24% was observed in the conjugate fraction with reductive amination. The influence of the conjugation process on the rPspA1 structure was assessed by circular dichroism. According to our results, both conjugation processes reduced the alpha-helical content of rPspA; reduction was more pronounced when the reaction between the polysaccharide capsule and rPspA1 was promoted between the carboxyl groups than the amine groups (46% and 13%, respectively). Regarding the immune response, both conjugates induced functional anti-rPspA1 and anti-PS6B antibodies. These results suggest that the secondary structure of PspA1, as well as its reactive groups (amine or carboxyl) involved in the linkage to PS6B, may not play an important role in eliciting a protective immune response to the antigens.

Streptococcus pneumoniae (pneumococcus) remains a leading cause of bacterial infectious diseases, particularly in children less than 2 years of age. About 800,000 children die annually due to pneumococcal disease, especially in emerging countries (1). The increasing number of antibiotic-resistant strains (2) and the severity of pneumococcal diseases make vaccination the most effective intervention.

Polysaccharide (PS) capsules are the main virulence factor of the pneumococci, which function by preventing phagocytosis and hampering bacterial clearance. Due to their high immunogenicity and importance in bacterial pathogenesis, PSs have been the antigens of choice in all current vaccines. The 23-valent pneumococcal polysaccharide vaccine (PPV23; Merck) has been shown to cover 80% to 90% of the serotypes responsible for invasive pneumococcal disease (IPD) in developed countries (3). According to a meta-analysis of randomized trials, the administration of PPV in immunocompetent adults can reduce the incidence of IPD and death due to pneumonia in this population by 71% and 32%, respectively. Conversely, pneumococcal polysaccharide vaccines are not effective in children under 2 years of age (4). The inefficacy of PS vaccines in this population has been attributed to the immaturity of the infant immune system in the expression of B cell receptors, including complement receptor type 2 (CR2) (5, 6).

Conjugation of PSs to carrier proteins converts it from a T cell-independent to a T cell-dependent antigen. As a T cell-dependent antigen, PS can raise a response with isotype switching, generation of memory cells, and a boosting effect (7).

The first pneumococcal conjugate vaccine (PCV) was licensed in 2000 as a 7-valent formulation (PCV7; Pfizer), which included capsular polysaccharides 4, 6B, 9V, 14, 18C, 19F, and 23F conju-

gated to the nontoxic variant of diphtheria toxin (CRM₁₉₇). In spite of the high degree of effectiveness of PCV7 in reducing pneumococcal diseases (8–12), recent reports have described an increase in the rate of disease caused by serotypes not included in this vaccine (13–15). The current pneumococcal vaccine strategy involves extending protection against emerging serotypes by increasing the valence to target additional serotypes (PCV13 [Pfizer], PCV10 [GlaxoSmithKline], PCV15 [in development by Merck]). An alternative to this trend could be the use of pneumococcal surface proteins as carriers conjugated to PSs from a few of the most common serotypes. The replacement of the same universal carrier proteins, such as tetanus toxoid (TT) or CRM₁₉₇, by a pneumococcal protein, besides broadening the vaccine coverage, would also prevent the impairment of immune responses caused by the excessive use of the same proteins in commercial vaccines (16, 17). In this study, we reinforce the use of pneumococcal surface protein A (PspA) as a promising carrier protein.

PspA is described to be an important pneumococcal virulence factor for inhibiting complement deposition (18, 19) and for pro-

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protecting pneumococci from killing by apolactoferrin (20). This protein is widely known to be immunogenic and protective (21, 22) and is present in all pneumococcal strains (23). According to sequence identities, PspA molecules have been classified into families and clades: family 1 (clades 1 and 2), family 2 (clades 3, 4, and 5), and family 3 (clade 6) (24). More than 90% of clinical isolates are distributed in family 1 or family 2 (25, 26).

Our group has previously demonstrated that conjugation of recombinant PspA (rPspA) to different PSs either maintains or increases its immunogenicity: (i) rPspA family 1, clade 1, conjugated to PS23F induced higher protection against lethal challenge than the nonconjugated rPspA (52), and (ii) rPspA family 2, clade 3, conjugated to polysaccharide serotype 14 (PS14) induced antibodies with a higher efficiency in complement deposition and higher opsonophagocytic activity than the nonconjugated protein (27). To extend these studies, PS6B was conjugated to rPspA family 1, clade 1, using two different methods of conjugation: the chemical linkage of PS6B either to the carboxyl groups or to the amine groups of rPspA. The focus of this study was to elucidate the influence of the method of conjugation on the efficiency of coupling PS6B to rPspA, on the secondary structure of the protein, and on the protective immune response induced against each antigen (PS6B and rPspA).

The improvement of conjugation yields also represents a current effort in the development of new conjugates. Improved conjugation yields increase the possibility of achieving an affordable manufacturing process. In the studies described herein, the method of conjugation through the carboxyl groups previously described by us (27, 52) was extended to the conjugation of PS6B with to rPspA clade 1 (rPspA1), and its efficiency was compared with that of reductive amination, a classical method used to obtain PCV7 and PCV13.

MATERIALS AND METHODS

Materials. Recombinant PspA clade 1 (rPspA1) and *S. pneumoniae* polysaccharide serotype 6B (PS6B) were produced in the Fermentation Laboratory of the Instituto Butantan (29–32). 1,8-Diaminooctane (OCT) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) were from Sigma-Aldrich (St. Louis, MO). *S. pneumoniae* strains (245/00 and 679/99) were generously supplied by Instituto Adolfo Lutz (São Paulo, Brazil). The strains were maintained as frozen stocks (−80°C) in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) with 10% glycerol.

Polysaccharide activation. Before activation, PS6B (10 mg/ml) was hydrolyzed with HCl (0.5 M) under agitation at 80°C for 1 h in a reflux system, followed by neutralization with NaOH to pH 7.5. Hydrolyzed PS6B was oxidized with NaIO₄ at a final concentration of 10 mM in 10 mM phosphate buffer (pH 7.5) for 30 min in the dark (3:2 molar ratio of the PS6B repeating unit to NaIO₄). The reaction was quenched by adding glycerol (10 eq). Oxidized PS6B was purified from the remaining glycerol and from low-molecular-weight oxidation products through chromatography using Sephadex G-25 gel filtration medium packed in an XK 26/40 column (GE Healthcare) and elution with 10 mM phosphate buffer (pH 7.5). The purified oxidized PS6B was lyophilized and resuspended to a final concentration of 10 mg/ml. The extent of oxidation was estimated by the bicinchoninic acid (BCA) colorimetric method (33) with glucose as the standard.

Polysaccharide derivatization. Oxidized PS6B (10 mg/ml) was incubated with OCT in a ratio of 100 mol of OCT per mol of aldehyde in PS. The reaction proceeded for 24 h in 10 mM phosphate buffer (pH 7.5). Sodium cyanoborohydride (NaBH₃CN) was then added at the same proportion used for OCT in order to reduce the Schiff's base generated and to

favor the formation of the PS6B-OCT product. Sodium borohydride in a ratio of 100 mol per mol of aldehyde in PS was dissolved in 2% NaOH (final volume, 100 µl) and added to the solution to stop the reaction. The product, PS6B-OCT, was purified by gel filtration chromatography using Sephadex G-25 packed in an XK 26/40 column (GE Healthcare) and elution with 10 mM phosphate buffer (pH 7.5). The extent of the reaction with OCT was estimated by the trinitrobenzenesulfonic acid (TNBS) method (34), using TNBS (Sigma-Aldrich) with OCT as the standard. After purification, the PS6B-OCT was lyophilized and stored at −20°C.

rPspA1 modification. rPspA1 (15 mg/ml) was treated with formaldehyde (5%) in the presence of a 5 M solution of sodium cyanoborohydride in 2% NaOH (10 µl per ml of reaction mixture) for 5 days at room temperature. Modified PspA1 (mPspA1) was purified by gel filtration chromatography using Sephadex G-25 packed in an XK 26/40 column (GE Healthcare) and eluted with 10 mM phosphate buffer (pH 7.5). The rPspA1 lysine content after the modification reaction was compared to that of rPspA1 by estimation using the TNBS method (34). After purification, mPspA1 was lyophilized and stored at −20°C.

PS6B-rPspA1 conjugation. (i) **Conjugation using DMT-MM.** mPspA1 (10 mg/ml) was activated with 0.1 M DMT-MM, followed by the addition of PS6B-OCT (15 mg/ml) (mass ratio, 1:1) in 10 mM phosphate buffer (pH 7.5) for 48 h. The PS6B-OCT-mPspA1 conjugate was dialyzed against 10 mM phosphate buffer (pH 7.5) and purified by hydrophobic chromatography in phenyl-Sepharose 6 Fast Flow High Sub packed in an XK 16/20 column (GE Healthcare), using descending gradient elution from 1 M to 0 M (NH₄)₂SO₄, starting in 50 ml and ending in 189 ml of the elution volume. The chromatography was performed using an ÄKTA Prime system (GE Healthcare) with a flow rate of 3 ml/min. The conjugate fraction was dialyzed against 1 mM sodium phosphate buffer (pH 7.5) and stored lyophilized at −20°C.

(ii) **Reductive amination method.** Oxidized PS6B was incubated with rPspA1 (recombinant PspA1 in its native form) for 15 days in a ratio of 1:1 (wt/wt) and final concentration of 5.5 mg/ml each in the presence of sodium cyanoborohydride (twice the PS mass) and 0.1% phenol. After 15 days, sodium borohydride was added to reduce the remaining aldehyde groups. The PS6B-rPspA1 conjugate was dialyzed against 10 mM sodium phosphate buffer (pH 7.5). The product was purified by hydrophobic chromatography in phenyl-Sepharose 6 Fast Flow High Sub packed in an XK 16/20 column (GE Healthcare), using descending gradient elution from 1 M to 0 M (NH₄)₂SO₄, starting in 50 ml and ending in 180 ml of the elution volume. The chromatography was performed using an ÄKTA Prime system (GE Healthcare) with a flow rate of 3 ml/min. The conjugate fraction was dialyzed against 1 mM sodium phosphate buffer (pH 7.5) and stored lyophilized at −20°C.

Analytical procedures. (i) **Measurement of PS.** The quantities of PS6B were measured by the phenol-sulfuric acid method (35) with a small modification: the reaction volumes were reduced 5 times, but the proportions of the reagents were maintained. Rhamnose was used as the standard.

(ii) **Measurement of protein.** The concentration of rPspA1 was assayed by the method of Lowry (36), using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin as the standard.

(iii) **Determination of molecular size.** The molecular size of hydrolyzed PS6B was determined in Sephacryl S-400 packed in an XK16/100 column (GE Healthcare), using 0.2 M NaCl as the mobile phase at 1 ml/min. The column was calibrated with dextrans (Sigma-Aldrich) of known sizes (2,000 kDa, 229 kDa, 70 kDa, 40 kDa, and 10 kDa).

CD analysis. The circular dichroism (CD) spectra were obtained on a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) at 20°C. The measurements were performed at wavelengths from 185 to 260 nm and intervals of 0.1 nm in a 0.1-cm-path cell. All samples were previously dialyzed against 10 mM sodium phosphate buffer, pH 7.5. The spectra presented are the averages of five scans, and the data obtained were reported as molar ellipticity (degrees-cm²-dmol^{−1}). A baseline measurement with sodium phosphate buffer was subtracted from each spectrum;

TABLE 1 PS6B and rPspA1 doses used in the immunization protocol^a

Group tested	Amt (μg)/dose	
	PS6B	rPspA1
Saline + adjuvant	0	0
Coadministered PS6B + rPspA1 (control 1)	15	45
PS6B-rPspA1 (test 1)	15	45
Coadministered PS6B + mPspA1 (control 2)	15	30
PS6B-OCT-mPspA1 (test 2)	15	30

^a The active carrier protein was rPspA1 (family 1, clade 1).

for each PS-protein conjugate, a measurement with the same amount of PS was also subtracted from the spectrum (the measurements for oxidized PS6B and PS6B-OCT were subtracted from the PS6B-rPspA1 and PS6B-OCT-mPspA1 spectra, respectively). The secondary structure deconvolution analyses were performed with Dichroweb software (37), using the CDSSTR algorithm (38).

Immunization procedures. BALB/c mice (female, 8 weeks old, and 6 per group) were obtained from the local breeding facility of the Universidade Federal de São Paulo (UNIFESP) and were immunized intraperitoneally (i.p.) on days 1, 14, and 28. The same dose of PS6B (15 μg) was established for both conjugates, and as the mass ratio of PS6B/rPspA1 varied in the conjugates produced, the rPspA1 dose also varied (described in detail in Table 1). The controls (coadministered compounds) were prepared to contain the same mass of protein and PS contained in their respective conjugates. All samples (500 μl per mouse) were prepared in 0.9% saline solution with 50 μg of Al(OH)₃ as the adjuvant. Sera were collected from mice on the 41st day by retro-orbital bleeding and kept at -20°C before use.

ELISA. Antibodies to rPspA1 were determined by conventional direct enzyme-linked immunosorbent assay (ELISA). PolySorp 96-well plates (Nunc) were coated with 0.1 μg per well of rPspA1 in 0.05 M sodium bicarbonate buffer (pH 9.6) overnight at 4°C. The plates were then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocked for 1 h at 37°C with PBS containing nonfat dried milk (10%). After this incubation time, the plates were washed with PBS-T and then incubated with serial dilutions of serum from individual mice in PBS for 1 h at 37°C. The plates were then washed with PBS-T and loaded with peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) in PBS. After a new incubation for 1 h at 37°C, the plates were washed and incubated for 15 min at room temperature in the dark with 40 μg of *o*-phenylenediamine (Sigma) and 0.5 μl of 3% hydrogen peroxide in 0.1 M citrate buffer (pH 5.0). The reaction was stopped by addition of 50 μl of 4 M sulfuric acid. The optical density was measured at 492 nm using an ELISA reader (Multiskan EX; Labsystems Uniscience) (39). The titer was defined as the dilution of serum that measured 0.1 at an optical density at 492 nm (OD₄₉₂).

Complement deposition assay. *S. pneumoniae* strain 245/00, bearing homologous PspA (serotype 14, PspA clade 1), was plated on blood agar, followed by growth in THY to an OD₆₀₀ of 0.4 to 0.5 (concentration, approximately 10⁸ CFU/ml). The samples were centrifuged at 4,000 × *g* for 3 min, and the pellets were washed once with PBS and resuspended in the same buffer. Sera from mice immunized with conjugates or control samples had their complement previously inactivated by heating at 56°C for 30 min and were added to the pneumococcus suspension at a final concentration of 10%; the mixture was incubated for 30 min at 37°C. After this incubation period, the bacteria were washed once with PBS and then incubated with 100 μl per well of 10% fresh-frozen normal mouse serum (NMS) from naive BALB/c mice in gelatin Veronal buffer (Sigma) for 30 min at 37°C. The bacteria were washed again with PBS, followed by another incubation with 100 μl of fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse complement C3 (MP Biomedicals) at a dilution of 1:500 on ice for 30 min in the dark. In the last step, the bacteria were washed twice with PBS and then resuspended in 1% formaldehyde for analysis in a FACSCanto flow cytometer (BD Biosciences).

Opsonophagocytic assay (OPA). *S. pneumoniae* strains 245/00 (serotype 14, PspA clade 1, used to evaluate the opsonic activity of anti-PspA antibodies) and 679/99 (serotype 6B, PspA clade 3, used to evaluate the opsonic activity of anti-PS6B antibodies) were grown in THY to an OD₆₀₀ of 0.4 to 0.5 (concentration, approximately 10⁸ CFU/ml) and harvested by centrifugation at 4,000 × *g* for 3 min. The pellets were washed once with PBS and resuspended in Hank's buffer (Invitrogen) containing 0.1% gelatin. Aliquots of bacteria containing 2.5 × 10⁶ CFU were incubated with heat-inactivated pooled test sera at a final dilution of 1:16 for 30 min at 37°C. Sera from mice injected with saline plus Al(OH)₃ were used as a control for all the assays. The samples were then incubated with 10% NMS diluted in opsono-buffer (Hank's buffer containing 0.1% gelatin) at 37°C for 30 min. Following incubation, the samples were washed once with PBS and then incubated with 4 × 10⁵ peritoneal cells diluted in opsono-buffer at 37°C for 30 min with shaking (200 rpm). Peritoneal cells were obtained as previously described (40). The reaction was stopped by cooling on ice for 5 min. Tenfold dilutions of the samples were plated on blood agar plates in triplicate. The plates were incubated at 37°C in a 5% CO₂ incubator, and the numbers of pneumococcal CFU recovered were counted after 20 h (40).

Statistical analysis. The significance of differences in the final pneumococcal counts in the protection studies was assessed using a one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. For all comparisons, a *P* value of <0.05 was considered to represent statistical significance.

RESULTS

Conjugation and purification of PS6B-rPspA1. Two different conjugates were synthesized: PS6B-OCT-mPspA1 (with an eight-carbon spacer molecule) and PS6B-rPspA1 (with no spacer molecule). PS6B-OCT-mPspA1 was prepared by the method developed in our laboratory (28). The steps of the conjugation process are represented in Fig. 1 and described above in detail in the experimental protocols. The acid hydrolysis of native PS6B reduced its size from 1,000 kDa to approximately 20 kDa. The aldehyde groups were obtained by a mild oxidation condition with NaIO₄ that resulted in 5 aldehydes per PS6B molecule (approximately 0.16 aldehyde per PS6B repeating unit). Eighty percent of the aldehydes inserted in the PS6B molecule were linked to the spacer molecule OCT, resulting in 4 OCT molecules per PS6B (approximately 0.128 OCT molecule per PS6B repeating unit). PS6B-rPspA1 was obtained by the currently used reductive amination method (41), using the same hydrolyzed and oxidized PS.

The rPspA1 employed in the DMT-MM-mediated conjugation was previously treated with formaldehyde in order to avoid intermolecular reactions and precipitation during conjugate synthesis. This modification process incorporates methyl groups in about 70% of the ε-amine groups of PspA lysine residues; methyl incorporation has been proven not to interfere with rPspA immunogenicity (27).

The conjugates were purified using hydrophobic interaction chromatography (HIC) (Fig. 2). PS6B, a hydrophilic molecule, did not bind to phenyl-Sepharose and eluted in the flowthrough fraction. rPspA1, which contains hydrophobic domains, interacted strongly with the resin and eluted after the end of the gradient. The conjugates combine the characteristics of both PS6B and rPspA and eluted in the last third of the decreasing ammonium sulfate gradient, allowing separation of the reagents and products. The conjugate elution was characterized by the coincidence of PS and protein detection in the same elution volume. Figure 2 shows the chromatograms of PS6B-OCT-mPspA (top) and PS6B-PspA (bottom) with 3 overlapping chromatograms each: (i) nonconju-

FIG 1 Conjugation steps. Native PS6B (1,000 kDa) has its size reduced to 20 kDa by acid hydrolysis. Then, aldehyde groups are produced by oxidation of the PS molecule. This reactive group (aldehyde) reacts directly with amine groups on rPspA by reductive amination (the method applied in commercial vaccines) or with amine groups present in OCT. In the second case, the product, PS6B-OCT, is subsequently reacted with carboxyl groups on mPspA, intermediated by DMT-MM, to form the conjugate. In order to increase the specificity of conjugation with PS6B-OCT, PspA's lysine was previously modified with formaldehyde (mPspA).

gated rPspA1, (ii) nonconjugated PS6B, and (iii) the conjugates. The relative amounts of the conjugated and nonconjugated PSs were measured from the column elution for calculation of the conjugation yields (42). The chromatograms shown for the nonconjugated compounds, rPspA1 and PS6B, are representative of a column loaded with 5 mg, and the chromatograms shown for the conjugates are representative of 25 mg of PS6B.

Using the methodology employing DMT-MM, $55.0\% \pm 6.0\%$ of the PS was in the PS6B-OCT-mPspA peak, whereas in the reductive amination method, $24.0\% \pm 2.6\%$ of the PS was associated with the conjugated PS6B-rPspA1 peak. The mass ratios of PS6B/rPspA1 obtained in the conjugates PS6B-OCT-mPspA1 and PS6B-rPspA1 were 1:2 and 1:3, respectively.

CD analysis. The effect of PS6B conjugation on rPspA secondary structure was analyzed by CD, comparing the CD spectra of conjugates and controls (rPspA1 and mPspA1). As shown in Fig. 3, there was a predominance of alpha-helix structures (78%) in rPspA1. After the treatment of rPspA1 with formaldehyde (mPspA1), the alpha-helix content changed from 78% (rPspA1) to 61% (mPspA1), a reduction of approximately 22%. The conju-

gation processes were also shown to disrupt the secondary structure of conjugated rPspA1 compared to that for the rPspA1 control: the reductive amination led to an alpha-helix reduction of 13%, while the process of conjugation by carboxamide formation reduced the alpha-helix content in the protein by 46% (a 22% reduction associated with treatment with formaldehyde and a 24% reduction associated with its conjugation with PS6B). The level of unordered structures increased proportionally to the reduction in the alpha-helix content of the protein.

Immunogenicity of conjugates. PS6B exhibits low immunogenicity in murine models (43), and its optimal dose ranges from 10 to 20 μg (44). In our immunization protocol, we used the conjugate dose equivalent to 15 μg of PS6B. This implied having different concentrations of rPspA1 per dose in the conjugate groups, since the PS6B/rPspA1 ratio varied in each conjugate. In order to compare the response induced against both antigens before and after conjugation, the controls (native PS6B plus rPspA1 or mPspA1) contained the same amount of PS6B and rPspA1 as the corresponding conjugate.

The anti-rPspA1 IgG titer induced by the conjugates and their

FIG 2 Purification of conjugates. Hydrophobic interaction chromatography (phenyl-Sepharose 6 Fast Flow High Sub) of PS6B-rPspA1 conjugate: PS6B-rPspA1 produced by reductive amination (top) and PS6B-OCT-mPspA1 produced by conjugation using DMT-MM (bottom). The chromatograms of PS6B and free rPspA1 are displayed in both panels. The amounts of free PS6B and conjugated PS6B loaded in the column were 5 mg and 25 mg, respectively. Elution was with a decreasing gradient from 1 M to 0 M $(\text{NH}_4)_2\text{SO}_4$. The absorbance (Abs) at 490 nm and the absorbance at 280 nm correspond to the measurements for PS6B and rPspA1, respectively.

control groups was measured by ELISA (Fig. 4A). The conjugate obtained by reductive amination (PS6B-rPspA1) induced the same anti-rPspA1 IgG titer as rPspA1 coadministered with PS6B (Fig. 4A). On the other hand, the conjugate synthesized by carboxamide formation (PS6B-OCT-mPspA1) displayed an anti-rPspA1 antibody titer higher than that induced with the coadministered antigens.

The functionality of these antibodies was evaluated by their ability to mediate complement deposition on the pneumococcal surface and their opsonophagocytic activity. The flow cytometry histograms of the complement deposition obtained when a serotype 14 strain bearing a PspA homologous to the conjugate was incubated with antisera from mice immunized with the conjugates were shown to be comparable to those obtained with the coadministered antigens (Fig. 4B). According to our results, the ability to induce opsonizing antibodies that mediate C3 complement deposition on the pneumococcus was preserved after conjugation, showing that the partial loss in the secondary structure of the rPspA molecule did not impair its ability to elicit opsonizing antibodies.

The opsonophagocytic activities exhibited by anti-PS6B antibodies and anti-rPspA antibodies in the sera of mice immunized with PS6B-OCT-mPspA1 and PS6B-rPspA1 were evaluated in two separate assays: (i) an assay using a pneumococcus serotype 6B strain carrying a PspA heterologous to the conjugate to assess the protective immunogenicity of anti-PS6B antibodies and (ii) an assay using a pneumococcus serotype 14 strain bearing a PspA homologous to the conjugate to measure the protective immunogenicity of anti-rPspA antibodies. Despite having different conformations, both conjugates were equally efficient in inducing opsonophagocytic antibodies against PS6B (shown by a significant reduction in the number of CFU recovered) (Fig. 5A). As expected, the sera of mice immunized with the nonconjugated rPspA1 or mPspA1 were efficient in reducing the number of CFU recovered compared to the efficiency for the sera of mice in the negative-control group immunized with saline and $\text{Al}(\text{OH})_3$ (Fig. 5B). The conjugation of the protein to PS6B did not result in the loss of opsonophagocytic activity of the anti-rPspA1 antibodies. On the contrary, the conjugation seemed to improve the protective activity of anti-rPspA antibodies, reducing bacterial survival

FIG 3 rPspA1 secondary structure following conjugation. The protein secondary structure was assessed by CD. rPspA1 was compared to rPspA1 after modification with formaldehyde (mPspA1) and to rPspA1 after conjugation to PS6B by reductive amination (PS6B-rPspA1) or by conjugation using DMT-MM (PS6B-OCT-mPspA1). CD spectra were obtained on a Jasco J-810 spectropolarimeter at 20°C. The measurements were performed at wavelengths from 185 to 260 nm and intervals of 0.1 nm in a 0.1-cm-path cell. The secondary structure deconvolution analysis was performed with Dichroweb software, using the CDSSTR algorithm.

and inducing the recovery of lower numbers of CFU ($P < 0.001$) (Fig. 5B).

DISCUSSION

In this study, we have compared the effect of two conjugation methodologies on the immunogenicity of both antigens present in the conjugate, PS6B and rPspA1. Both methods start by oxidation of the vicinal hydroxyls of the polysaccharides, creating aldehyde

groups. In the classical reductive amination method, the aldehyde is bound directly to an ϵ -amine group of lysine in the rPspA1 molecule. In the DMT-MM-mediated method, the aldehyde was first coupled with OCT and then the free amine group of derivatized PS (PS-OCT) reacted with the carboxyl groups of mPspA1 (41). The reaction mechanism of conjugation mediated by DMT-MM is shown in Fig. 6: the carboxyl group in the carrier protein (A) is activated by DMT-MM (B), resulting in an acyloxytriazine intermediate (A-B). The NH_2 , a nucleophilic group from PS-OCT (C), reacts with the intermediate (A-B) through a carboxamide linkage, generating the conjugate (A-C). The use of this reagent in the production of a conjugate vaccine was first proposed by our laboratory (29). The majority of the conjugation methods that are based on the reaction of carboxyl groups employ 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in their activation step. We introduced DMT-MM as a more efficient activating reagent than EDC due to its higher stability in aqueous solution, especially when using phosphate buffer (45). Studies have shown that the use of DMT-MM in coupling PS with small bioactive molecules or with microspheres resulted in higher reaction yields than conventional methodologies (46, 47). Likewise, as shown by the HIC chromatograms of the conjugates, the amount of PS6B conjugated to rPspA1 is higher in the method that uses DMT-MM-mediated conjugation ($55.0\% \pm 6.0\%$) than in the reductive amination ($24.0\% \pm 2.6\%$). When using adipic acid dihydrazide (ADH; a currently used spacer with 6 molecules) instead of OCT, no difference in the yield of the reaction between the PS and protein was observed (results not shown).

Serotype 6B strains are epidemiologically important, and PS6B is in the currently licensed PCVs. PS6B appears at double the concentration of the other serotypes in the 7-valent and 13-valent formulations. Despite being present at a higher dose, the levels of antibodies induced against PS6B are the lowest among the levels of

FIG 4 Anti-PspA immune response. (A) IgG antibody titer to rPspA1. Individual serum samples from mice ($n = 6$) immunized i.p. with rPspA1 conjugated to PS6B by reductive amination (PS6B-rPspA1) or with mPspA1 conjugated to PS6B-OCT (PS6B-OCT-mPspA1) were analyzed by ELISA and compared by one-way ANOVA with Tukey's multiple-comparison test. Sera from mice immunized with the respective coadministered components or with saline plus $\text{Al}(\text{OH})_3$ were used as controls. Asterisks indicate statistically significant differences (***, $P < 0.0001$). ns, nonsignificant differences. The results for all groups were significantly different from those for the saline-treated group ($P < 0.0001$). (B) Complement deposition on *S. pneumoniae* bacteria. An example of a flow cytometry histogram for C3 deposition is shown. *S. pneumoniae* strain 245/00 (serotype 14 and PspA clade 1) was incubated with sera from mice immunized with PspA clade 1 conjugated to PS6B or to PS6B-OCT. Sera from mice immunized with the respective coadministered components or with saline plus $\text{Al}(\text{OH})_3$ were used as controls.

FIG 5 Opsonophagocytic assay. Pneumococcal strain 679/99 (PspA clade 3, serotype 6B, used to test the opsonic activity of anti-PS6B antibodies [Ab]) (A) and pneumococcal strain 245/00 (PspA clade 1, serotype 14, used to test the opsonic activity of anti-PspA1 antibodies) (B) were incubated with the sera from mice immunized with PS6B-rPspA1 or with PS6B-OCT-mPspA1 and a complement source. The opsonized pneumococci were incubated with peritoneal cells and plated on blood agar plates. Sera from mice immunized with saline plus $\text{Al}(\text{OH})_3$ or with PS6B coadministered with rPspA1 or mPspA1 were used as controls. The numbers of CFU recovered after 20 h were compared by one-way ANOVA with Tukey's multiple-comparison test. The lines on the graph represent means. Asterisks indicate statistically significant differences (**, $P < 0.001$; ***, $P < 0.0001$). ns, nonsignificant differences.

antibodies induced against the serotypes included in the PCVs (48). Regardless of the antibody concentrations, PS6B induces a protective response, demonstrated by the ability of the anti-PS6B antibodies to induce opsonophagocytosis of pneumococci (48). Due to its correlation with protection, we compared the effectiveness of our conjugates by OPA. The serum of mice immunized with the conjugates showed antibodies equally able to opsonize and mediate the phagocytosis of *S. pneumoniae* serotype 6B expressing a heterologous PspA. This demonstrates that the changes in chemical structure of the conjugates (spacer molecules and different types of linkages between PS and the carrier protein) did not influence the immune response induced against the PS.

The incorporation of PspA as a carrier protein in PCVs more than confers a T cell-dependent identity to the PS, and PspA is expected to act as an immunogenic antigen. Therefore, we analyzed the effect of the conjugation reaction on the secondary structure of the protein and on the immune response induced. The recombinant fragment of PspA used in this study contains the two

main regions related to protection: the N-terminal alpha-helical domain and the proline-rich region. The N-terminal alpha-helical region of PspA is long known for its protective potential (22–24, 49, 50). Recently, the proline region has also been shown to induce protection against pneumococcal infections (51). The secondary structure of conjugated rPspA1 was assessed by CD, and rPspA1 and mPspA1 molecules were used as controls. According to our results, both conjugates showed a reduction in the content of the alpha-helical structure and an increased amount of unordered structure in comparison to the rPspA1 molecule. The process of conjugation using DMT-MM led to a 31% reduction in alpha-helical content in comparison to that of its immediate nonconjugated precursor, mPspA1; a global reduction of 46% was obtained when losses due to both the rPspA1 modification and the conjugation are considered. The reductive amination had a minor effect on the alpha-helical content, leading to a reduction of only 13%.

The protective immunity conferred by immunization with PspA has usually been assessed in pneumococcal disease models.

FIG 6 Mechanism of conjugation mediated by DMT-MM: the carboxyl group (A) is activated by DMT-MM (B) and an acyloxytriazine intermediate is obtained (A-B). This intermediate (A-B) is susceptible to attack by a nucleophile, leading to the formation of A-C.

A restricted repertoire of pneumococcal strains is virulent in murine models, and these strains usually bear capsular polysaccharides 3, 6A, and 6B (21). In the present case, the selection of a strain virulent for mice mainly relies on strains bearing capsular type 3. The selection of a strain bearing serogroup 6 would impair the analysis of the immune response induced by PspA. The strains carrying PspA clade 1 and serotype 3 were shown to be highly pathogenic, causing rapid sepsis and death in the mice, while tests using serotypes different from serotypes 3, 6A, and 6B did not cause disease in the animals (data not shown). In the absence of a suitable pneumococcal disease model, the OPA was the assay of choice for evaluating the functional activity of anti-PspA antibodies (27, 40).

Notably, both conjugation processes preserved PspA's antigenic properties, including the ability to induce antibodies capable of mediating complement deposition and phagocytosis. These results would indicate that the primary sequence of amino acid residues in rPspA1, rather than its secondary structure, is probably associated with the induction of protective antibodies.

Our main goals with this study were to investigate whether rPspA1 could act as a carrier protein for PS6B and whether the conjugation would disrupt rPspA1's structure, affecting its immunogenicity. We observed that, when conjugated, rPspA1 induced lower levels of antibodies, although it had higher opsonophagocytic activity than when it was nonconjugated (Fig. 5B). The ratio of the opsonophagocytic activity per unit of antibody titer to rPspA1 was 15% higher for the conjugated rPspA1 than for the coadministered rPspA1 (data from Fig. 4A and 5A). A hypothesis for this observation is that surface PspA1, contrary to rPspA1, interacts with other pneumococcal surface components, including the capsular polysaccharide, and the charge distribution of conjugated rPspA1 (positively charged protein and negatively charged PS) more closely resembles that of the conformational structure of PspA expressed on the bacterial surface.

In conclusion, despite the fact that the circular dichroism analysis has shown that the conjugation alters the secondary structure of rPspA1, the immunological assays have demonstrated that these alterations do not affect its ability to induce a protective immune response. Furthermore, the conjugation strategy using different chemical linkages does not seem to impair the immunogenicity of rPspA1 or PS6B and, consequently, does not impose an obstacle to implementation of the more economical methodology. Therefore, our results support the use of rPspA1 as an antigenic carrier protein and reinforce the use of DMT-MM-mediated conjugation as a valuable strategy to be considered in conjugation processes.

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REFERENCES

- O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T. 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 374:893–902.
- Linares J, Ardanuy C, Pallares R, Fenoll A. 2010. Changes in antimicrobial resistance, serotypes and genotypes in *Streptococcus pneumoniae* over a 30-year period. *Clin. Microbiol. Infect.* 16:402–410.
- Fedson DS, Nicolas-Spony L, Klemets P, van der Linden M, Marques A, Salleras L, Samson SI. 2011. Pneumococcal polysaccharide vaccination for adults: new perspectives for Europe. *Expert Rev. Vaccines* 10:1143–1167.
- Cornu C, Yzebe D, Leophonte P, Gaillat J, Boissel JP, Cucherat M. 2001. Efficacy of pneumococcal polysaccharide vaccine in immunocompetent adults: a meta-analysis of randomized trials. *Vaccine* 19:4780–4790.
- Griffioen AW, Rijkers GT, Janssens-Korpela P, Zegers BJ. 1991. Pneumococcal polysaccharides complexed with C3d bind to human B lymphocytes via complement receptor type 2. *Infect. Immun.* 59:1839–1845.
- Timens W, Boes A, Rozeboom-Uiterwijk T, Poppema S. 1989. Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *J. Immunol.* 143:3200–3206.
- Guttormsen HK, Sharpe AH, Chandraker AK, Brigtsen AK, Sayegh MH, Kasper DL. 1999. Cognate stimulatory B-cell–T-cell interactions are critical for T-cell help recruited by glycoconjugate vaccines. *Infect. Immun.* 67:6375–6384.
- Redelings MD, Sorvillo F, Simon P, Mascola L. 2005. Declining early childhood mortality from invasive pneumococcal disease: the impact of vaccination. *Arch. Pediatr. Adolesc. Med.* 159:195–196.
- Roush SW, Murphy TV. 2007. Historical comparisons of morbidity and mortality for vaccine-preventable diseases in the United States. *JAMA* 298:2155–2163.
- Kellner JD, Vanderkooi OG, MacDonald J, Church DL, Tyrrell GJ, Scheifele DW. 2009. Changing epidemiology of invasive pneumococcal disease in Canada, 1998–2007: update from the Calgary-area *Streptococcus pneumoniae* research (CASPER) study. *Clin. Infect. Dis.* 49:205–212.
- Ruckinger S, van der Linden M, Reinert RR, von Kries R, Burckhardt F, Siedler A. 2009. Reduction in the incidence of invasive pneumococcal disease after general vaccination with 7-valent pneumococcal conjugate vaccine in Germany. *Vaccine* 27:4136–4141.
- Grijalva CG, Nuorti JP, Arbogast PG, Martin SW, Edwards KM, Griffin MR. 2007. Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis. *Lancet* 369:1179–1186.
- Chibuk TK, Robinson JL, Hartfield DS. 2010. Pediatric complicated pneumonia and pneumococcal serotype replacement: trends in hospitalized children pre and post introduction of routine vaccination with pneumococcal conjugate vaccine (PCV7). *Eur. J. Pediatr.* 169:1123–1128.
- Leach AJ, Morris PS, McCallum GB, Wilson CA, Stubbs L, Beissbarth J, Jacups S, Hare K, Smith-Vaughan HC. 2009. Emerging pneumococcal carriage serotypes in a high-risk population receiving universal 7-valent pneumococcal conjugate vaccine and 23-valent polysaccharide vaccine since 2001. *BMC Infect. Dis.* 9:121. doi:10.1186/1471-2334-9-121.
- Munoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R. 2008. Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. *Clin. Infect. Dis.* 46:174–182.
- Borrow R, Dagan R, Zepp F, Hallander H, Poolman J. 2011. Glycoconjugate vaccines and immunointeractions and implication for vaccination schedules. *Expert Rev. Vaccines* 10:1621–1631.
- Dagan R, Poolman J, Siegrist CA. 2010. Glycoconjugate vaccines and immune interference: a review. *Vaccine* 28:5513–5523.
- Ren B, Szalai AJ, Thomas O, Hollingshead SK, Briles DE. 2003. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. *Infect. Immun.* 71:75–85.
- Tu AH, Fulgham RL, McCrory MA, Briles DE, Szalai AJ. 1999. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. *Infect. Immun.* 67:4720–4724.
- Shaper M, Hollingshead SK, Benjamin WH, Jr, Briles DE. 2004. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. *Infect. Immun.* 72:5031–5040.
- Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, Ferguson LM, Nahm MH, Nabors GS. 2000. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J. Infect. Dis.* 182:1694–1701.
- Wu HY, Nahm MH, Guo Y, Russell MW, Briles DE. 1997. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *J. Infect. Dis.* 175:839–846.

23. Crain MJ, Waltman WD, II, Turner JS, Yother J, Talkington DF, McDaniel LS, Gray BM, Briles DE. 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. *Infect. Immun.* 58:3293–3299.
24. Hollingshead SK, Becker R, Briles DE. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect. Immun.* 68:5889–5900.
25. Hollingshead SK, Baril L, Ferro S, King J, Coan P, Briles DE. 2006. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. *J. Med. Microbiol.* 55:215–221.
26. Pimenta FC, Ribeiro-Dias F, Brandileone MC, Miyaji EN, Leite LC, Sgambatti de Andrade AL. 2006. Genetic diversity of PspA types among nasopharyngeal isolates collected during an ongoing surveillance study of children in Brazil. *J. Clin. Microbiol.* 44:2838–2843.
27. Santamaria R, Goulart C, Perciani CT, Barazzone GC, Carvalho R, Goncalves VM, Leite LC, Tanizaki MM. 2011. Humoral immune response of a pneumococcal conjugate vaccine: capsular polysaccharide serotype 14-lysine modified PspA. *Vaccine* 29:8689–8695.
28. Barazzone GC, Perciani CT, Raw I, Tanizaki MM. July 2011. Método de conjugação de polissacarídeo capsular a uma proteína carregadora, para uso como antígeno vacinal contra bactérias encapsuladas, utilizando o reagente cloreto de 4-(4,6-dimetoxi-1,3,5-triazin-2-il)-4-metilmorfolino (DMT-MM). Brazilian patent PI0904528-7.
29. Barazzone GC, Carvalho RJ, Kraschowetz S, Horta ACL, Sargo C, Silva AJ, Tanizaki MM, Cabrera-Crespo J, Gonçalves VM. 2011. Production and purification of recombinant fragment of pneumococcal surface protein A (PspA) in *Escherichia coli*. *Proc. Vaccinol.* 4:27–35.
30. Carvalho RJ, Cabrera-Crespo J, Tanizaki MM, Gonçalves VM. 2012. Development of production and purification processes of recombinant fragment of pneumococcal surface protein A in *Escherichia coli* using different carbon sources and chromatography sequences. *Appl. Microbiol. Biotechnol.* 94:683–694.
31. Silva M, Cabrera-Crespo J, Sbrogio-Almeida ME, Miyaji EN, Ho PL, Leite LC, Lopes AP. 2007. Optimizing expression of *Streptococcus pneumoniae* surface protein a, PspA: serocross-reactivity within families of antisera induced against clades 1 and 3. *Mol. Biotechnol.* 37:146–154.
32. Gonçalves VM, Takagi M, Carmo TS, Albani SMF, Pinto JV, Zangrolami TC, Giordano RC, Tanizaki MM, Cabrera-Crespo J. 2007. Simple and efficient method of bacterial polysaccharides purification for vaccines production using hydrolytic enzymes and tangential flow ultrafiltration, p 250–257. *In* Mendez-Vilas A (ed), *Communicating current research and educational topics and trends in applied microbiology*. Formatex, Badajoz, Spain.
33. Tyllianakis PE, Kakabakos SE, Evangelatos GP, Ithakissios DS. 1994. Direct colorimetric determination of solid-supported functional groups and ligands using bincinonic acid. *Anal. Biochem.* 219:335–340.
34. Qi XY, Keyhani NO, Lee YC. 1988. Spectrophotometric determination of hydrazine, hydrazides, and their mixtures with trinitrobenzenesulfonic acid. *Anal. Biochem.* 175:139–144.
35. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350–356.
36. Fryer HJ, Davis GE, Manthorpe M, Varon S. 1986. Lowry protein assay using an automatic microtiter plate spectrophotometer. *Anal. Biochem.* 153:262–266.
37. Whitmore L, Wallace BA. 2008. Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. *Biopolymers* 89:392–400.
38. Compton LA, Johnson WC, Jr. 1986. Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. *Anal. Biochem.* 155:155–167.
39. Darrieux M, Moreno AT, Ferreira DM, Pimenta FC, de Andrade AL, Lopes AP, Leite LC, Miyaji EN. 2008. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *J. Med. Microbiol.* 57:273–278.
40. Goulart C, Darrieux M, Rodriguez D, Pimenta FC, Brandileone MC, de Andrade AL, Leite LC. 2011. Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. *Vaccine* 29:1634–1642.
41. Anderson P, Pichichero ME, Insel RA. 1985. Immunogens consisting of oligosaccharides from the capsule of *Haemophilus influenzae* type b coupled to diphtheria toxoid or the toxin protein CRM197. *J. Clin. Invest.* 76:52–59.
42. Lee CH, Kuo WC, Beri S, Kapre S, Joshi JS, Bouveret N, LaForce FM, Frasch CE. 2009. Preparation and characterization of an immunogenic meningococcal group A conjugate vaccine for use in Africa. *Vaccine* 27:726–732.
43. Fairchild RL, Braley-Mullen H. 1983. Characterization of the murine immune response to type 6 pneumococcal polysaccharide. *Infect. Immun.* 39:615–622.
44. Chu RS, McCool T, Greenspan NS, Schreiber JR, Harding CV. 2000. CpG oligodeoxynucleotides act as adjuvants for pneumococcal polysaccharide-protein conjugate vaccines and enhance antipolysaccharide immunoglobulin G2a (IgG2a) and IgG3 antibodies. *Infect. Immun.* 68:1450–1456.
45. Gilles MA, Hudson AQ, Borders CL, Jr. 1990. Stability of water-soluble carbodiimides in aqueous solution. *Anal. Biochem.* 184:244–248.
46. Farkas P, Bystricky S. 2007. Efficient activation of carboxyl polysaccharides for the preparation of conjugates. *Carbohydr. Polymers* 68:187–190.
47. Schlottmann SA, Jain N, Chirmule N, Esser MT. 2006. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. *J. Immunol. Methods* 309:75–85.
48. Vesikari T, Wysocki J, Chevallier B, Karvonen A, Czajka H, Arsène JP, Lommel P, Dieussaert I, Schuerman L. 2009. Immunogenicity of the 10-valent pneumococcal non-typeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine. *Pediatr. Infect. Dis. J.* 28(4 Suppl):S66–S76.
49. McDaniel LS, Ralph BA, McDaniel DO, Briles DE. 1994. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. *Microb. Pathog.* 17:323–337.
50. McDaniel LS, Sheffield JS, Swiatlo E, Yother J, Crain MJ, Briles DE. 1992. Molecular localization of variable and conserved regions of pspA and identification of additional pspA homologous sequences in *Streptococcus pneumoniae*. *Microb. Pathog.* 13:261–269.
51. Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, Hollingshead SK. 2010. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. *Infect. Immun.* 78:2163–2172.
52. Csordas FC, Perciani CT, Darrieux M, Goncalves VM, Cabrera-Crespo J, Takagi M, Takagi M, Sbrogio-Almeida ME, Leite LC, Tanizaki MM. 2008. Protection induced by pneumococcal surface protein A (PspA) is enhanced by conjugation to a *Streptococcus pneumoniae* capsular polysaccharide. *Vaccine* 26:2925–2929.

Humoral immune response of a pneumococcal conjugate vaccine: Capsular polysaccharide serotype 14–Lysine modified PspA

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ABSTRACT

Polysaccharide–protein conjugates are so far the current antigens used for pneumococcal vaccines for children under 2 years of age. In this study, pneumococcal surface protein A (PspA) was used as a carrier protein for pneumococcal capsular polysaccharide serotype 14 as an alternative to broaden the vaccine coverage. PspA was modified by reductive amination with formaldehyde in order to improve the specificity of the reaction between protein and polysaccharide, inhibiting polymerization and the gel formation reaction. In the synthesis process, the currently used activator, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDAC) was substituted for 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). BALB/c mice were immunized with either the PS14–mPspA conjugate or the co-administered components in a three dose regimen and sera from the immunized animals were assayed for immunity induced against both antigens: PS14 and mPspA. Modification of more than 70% of lysine residues from PspA (mPspA) did not interfere in the immune response as evaluated by the anti-PspA titer and C3 complement deposition assay. Sera of mice immunized with conjugated PS14–mPspA showed similar IgG titers, avidity and isotype profile as compared to controls immunized with PspA or mPspA alone. The complement deposition was higher in the sera of mice immunized with the conjugate vaccine and the opsonophagocytic activity was similar for both sera. Conjugation improved the immune response against PS14. The anti PS14 IgG titer was higher in sera of mice immunized with the conjugate than with co-administered antigens and presented an increased avidity index, induction of a predominant IgG1 isotype and increased complement deposition on a bacteria with a surface serotype 14. These results strongly support the use of PspA as carrier in a conjugate vaccine where both components act as antigens.

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1. Introduction

Streptococcus pneumoniae is a major cause of pneumonia in infants and in the elderly. Following the widespread use of Haemophilus influenzae b (Hib) vaccination, pneumococcal infection is also the most common cause of bacterial meningitis in developed countries [1]. The first commercialized pneumococcal conjugate vaccine, PCV7, composed of capsular polysaccharides from seven different serotypes conjugated to the carrier protein CRM197 has provided convincing support for the effectiveness in preventing invasive pneumococcal diseases in young children [2–4]. Other new conjugate vaccines, a 10-valent and a 13-valent,

are now also available with improvement in the efficacy due to the inclusion of higher numbers of capsular polysaccharides.

Besides the high cost of these vaccines, other problems have been considered subject of concern: (i) among more than 92 different serotypes, 23 serotypes are considered the most worldwide prevalent ones and, since the serotype prevalence varies among regions, it is very hard to obtain one vaccine with high worldwide coverage, (ii) since most of the conjugate vaccines use tetanus toxoid, diphtheria toxoid or CRM197 as carrier, a multivalent conjugate as polyvalent pneumococcal vaccine might have a risk of immune interference [5,6], and (iii) 10 years after the widespread use of PCV7, emergence of non vaccine serotypes have been noticed [7,8].

In order to circumvent these problems with polysaccharide–protein conjugates, protein vaccines have been tested as alternatives. Among these proteins, Pneumococcal surface protein A (PspA) [9], Pneumococcal surface protein C (PspC) [10], Pneumolysin (Ply) and its derivatives [11], serine-threonine kinase (StkP) [12] have been successfully tested in laboratory

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animals. Despite all the encouraging results, it is still not clear whether protein vaccines will be able to induce compatible levels of protection as PS conjugate vaccines. The use of pneumococcal proteins exposed on the surface of the pneumococcus as carriers in a conjugate vaccine, may solve the need to use a high number of PS of different serotypes and could improve its efficacy and coverage. For this purpose, a method of conjugation that does not interfere in the protein immunogenicity should be selected. The capsular polysaccharide of the most prevalent serotypes and a conserved protein which induces protective antibodies should be selected. Serotype 14 is one of the most prevalent serotypes worldwide in developed and developing countries [13,14] and for this reason it is present in all pneumococcal vaccines. PspA is an important protein associated to pneumococcal virulence, it is exposed on the pneumococcal surface and its most well studied function is the inhibition of complement deposition on the bacterial surface [15]. Therefore PspA could be a good candidate as carrier protein in a conjugate vaccine. We describe here the synthesis of PS serotype 14 (PS14) conjugated to a modified PspA protein (mPspA) and the humoral immune response induced against this conjugate.

2. Material and methods

2.1. Materials

PS serotype 14 was obtained from The American Type Culture Collection (ATCC). Pneumococcal strains were obtained from Serviço de Bacteriologia, Instituto Adolfo Lutz, São Paulo, Brazil and from Universidade Federal de Goiás, Goiânia, Brazil. Sodium periodate, sodium borohydride, sodium cyanoborohydride (NaBH_3CN), adipic acid dihydrazide (ADH), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and 2,4,6-trinitrobenzenesulfonic acid (TNBS), goat anti-mouse IgG1, IgG2a, IgG2b, IgG3 horseradish peroxidase labeled antibodies were purchased from Sigma Chemical Company (St. Louis, MO). Bicinchoninic acid (BCA) was from Pierce (Rockford, IL). Sephadex G-25, Sephacryl S-400 and Phenyl Sepharose were from GE Healthcare.

2.2. Recombinant PspA

A PspA fragment family 2 clade 3 containing 6His was cloned in the expression vector pET-37b(+) and produced in *Escherichia coli* BL21(DE3) according to methods previously published [16] and purified through three chromatographic steps, Q-Sepharose, Metal chelating Sepharose loaded with Ni^{+2} and SP-Sepharose [17].

2.3. Reaction of PspA with formaldehyde

The PspA protein (20 mg/mL) was reacted with 2% formaldehyde (Merck – 37%) and 10 μL of a 5 M solution of sodium cyanoborohydride mL^{-1} of reaction in 1 M sodium hydroxide for 7 days at 25 °C in phosphate buffer 10 mM pH 7.5. The excess of both reagents was eliminated by dialysis against the same buffer. The ϵ -amino-groups which had not reacted were quantified by the TNBS method (see below).

2.4. Preparation of PS14–mPspA conjugate

PS14 (10 mg/mL) was hydrolyzed with HCl (0.5 M) under agitation at 80 °C during 30 min in a reflux system followed by neutralization with NaOH to achieve pH 7.5. The hydrolyzed PS14 with about 50 kDa (10.0 mg/mL) was oxidized with NaIO_4 (10 mM) in phosphate buffer 10 mM pH 7.5 for 30 min in the dark and quenched adding glycerol (10 eq.). The reaction mixture was diafiltered in the LabScale equipment (Millipore) using a 5 kDa cut-off

membrane (Pellicon XL, Millipore) against water. Oxidized PS14 was incubated with ADH in a molar ratio of 50 mol of ADH/mol of aldehyde and sodium cyanoborohydride (NaBH_3CN) 5 M in sodium hydroxide 0.2% (w/v) at 50 mol PS/mol of aldehyde. This reaction was maintained for 24 h in phosphate buffer 10 mM (pH = 7.5) and the reaction was quenched with 5 M sodium borohydride in 0.2% NaOH in a molar ratio of 10:1 (NaBH_4 : PS). The product, PS14-ADH, was purified by gel filtration chromatography using Sephadex G-25 in water. For the conjugation reaction, mPspA (15 mg/mL) was previously activated with 0.05 M of DMT-MM followed by the addition of PS (mass ratio of 1:1). The reaction occurred in phosphate buffer 10 mM with NaCl 0.3 M (pH 7.5) during 24 h. The product was dialyzed and purified by hydrophobic chromatography in Phenyl Sepharose 6Fast Flow High Sub packed in a XK 16/20 column (GE Healthcare) and eluted in a gradient of 1–0 M ammonium sulfate.

2.5. Analyticals

PS14 was quantified by the Phenol Sulfuric method [18]. The extension of oxidation was estimated by the colorimetric method using BCA [19]. The extension of the reaction with ADH as well as the lysine ϵ -amino group were estimated by TNBS method [20] –using ADH and lysine, respectively, as standard.

2.6. Immunization of mice with conjugate PS14–mPspA

Female BALB/c mice were immunized intraperitoneally with PS14–mPspA conjugate and the controls: PS14 + mPspA, PspA and saline. The vaccines contained 2.5 μg of PS14 and 5.5 μg of mPspA, or PspA in saline solution were mixed with 200 μg of $\text{Al}(\text{OH})_3$. The animals received three doses of the immunization at 30-day intervals. Sera were collected from mice at 29, 59, and 89 days by retro-orbital bleeding and kept at –20 °C before use.

2.7. ELISA to measure total antibody, avidity and isotype profile

Total antibody: ELISA 96-well microtiter plates (Nunc MaxiSorp™; Nalgen Nunc International, Rochester, NY) were coated with 5 μg /well of PS14 or 0.1 μg /well of PspA in PBS (pH 7.2) for 48 h or overnight at 4 °C, respectively. Plates were washed three times with PBS and 0.05% Tween 20 (PBS-T) and were blocked with PBS and 10% of skim milk for 1 h at 37 °C. Eight-fold dilutions of serum samples in PBS and 5% skim milk were then added for 2 h at 37 °C for anti-PS14 or 1 h at 37 °C for anti-PspA, and plates were washed three times with PBS-T. Peroxidase-conjugated polyclonal goat anti-mouse IgG (1:1000) was then added, and plates were incubated at 37 °C for 2 h (PS14) or 1 h (PspA). Plates were washed three times with PBS-T followed by addition of substrate *o*-phenylenediamine dihydrochloride in citrate buffer (pH 5.0) with 5 μL /mL of 10% hydrogen peroxide for 15 min in the dark. The enzyme reaction was quenched by adding 4 M H_2SO_4 . Plates were read at 492 nm on a Multiskan EX ELISA reader (Labsystems Uniscience, São Paulo, S.P.). Titers were calculated by using the dilution resulting in an absorbance value of 0.1 at 492 nm. Sera from individual animals were tested separately and had the absorbance value of saline samples subtracted. The statistical treatment was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test for comparison of groups. The significance level was $p < 0.05$.

Avidity assay: IgG avidity was determined by ELISA in quadruplicate, with the inclusion of one additional step to the protocol described above: after the addition of the serum and the wash step, 100 μL of KSCN 1.5 M dissolved in PBS was added to one half of the plate and PBS was added to the other half. The avidity index AI was calculated according to the previously described method [21].

Isotype profile: the IgG isotyping was performed using the same protocol for total antibody measurement with the following modification: after incubation with serum of immunized mice, affinity-purified goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 horseradish peroxidase labeled antibodies diluted 1:1000 were used.

2.8. Complement deposition assay and opsonophagocytic assay

Complement deposition was first evaluated in the sera of mice immunized with free PspA or mPspA: 3 pneumococcal strains bearing PspA family 2, clade 3 were used, strains StP30 (PS14/PspA3), P539 (PS19F/PspA3) and P122 (PS10A/PspA3). Complement deposition was also evaluated in the sera of mice immunized with mPspA conjugated to PS14 and the co-administrated control (mPspA+PS14). In this case, a strain of serotype 3 bearing PspA3 was used, strain P275/97-PS 3. To evaluate the immune response induced against PS14, two strains of serotype 14, clade 1, were used, strains St245/00 (PS14/PspA1) and P630 (PS14/PspA1). All pneumococcal strains were grown in THY up to 10^8 CFU/mL (optical density of 0.4–0.5) and harvested by centrifugation at $2000 \times g$ for 3 min. The pellets were washed once, resuspended in PBS, incubated with pooled heat-inactivated (56°C for 30 min) sera from immunized mice at a final concentration of 10% for 30 min at 37°C . Bacteria were then washed once with PBS, resuspended in 90 μL of gelatin in Veronal buffer and incubated with 10% normal mouse serum (from BALB/c mice) at 37°C for 30 min. After washing with PBS, the samples were incubated with 100 μL of FITC-conjugated goat antiserum to mouse complement C3 (MP Biomedicals) at a dilution of 1:500 on ice for 30 min in the dark, washed twice with PBS, resuspended in 1% formaldehyde, and stored at 4°C in the dark until analysis with a FACSCanto (BD Biosciences).

Opsonophagocytic assay was performed according to that previously described [22]. Briefly, *S. pneumoniae* serotype 6B strain 679/99 (PS6B/PspA3/), expressing PspA3 and polysaccharide 6B, was grown in THY up to a concentration of 10^8 CFU/mL (optical density of 0.4–0.5) and harvested by centrifugation at $2000 \times g$ for 3 min. The pellets were washed once with PBS, resuspended in opsono buffer, and aliquots containing 2.5×10^6 CFU were incubated with heat-inactivated sera from mice immunized with conjugate vaccine and controls at a final dilution of 1:8 at 37°C for 30 min. After the second wash with PBS, the samples were incubated with 10% normal mouse serum (NMS) at 37°C for 30 min. The samples were then washed once with PBS and incubated with 4×10^5 stimulated peritoneal cells from BALB/c mice diluted in opsono buffer at 37°C for 45 min with shaking (220 rpm). Peritoneal macrophages were assessed 48 h after i.p. injection with 10 μg of Concanavalin A from *Canavalia ensiformis* (ConA, Sigma) and their peritoneal cavities washed with 5 mL of ice-cold PBS. The reaction was stopped by incubation on ice for 5 min. Ten-fold dilutions of the samples were performed and 10 μL aliquots of each dilution were plated on blood agar plates. The plates were incubated at 37°C , 5% CO_2 and the pneumococcal CFU recovered counted after 18 h.

3. Results

3.1. Synthesis of the conjugate PS serotype 14-PspA

The conjugate was synthesized by the method developed in our laboratory [23] with one modification – use of DMT-MM instead EDAC. The method consists in the following steps: (a) PS14 hydrolysis, (b) PS oxidation, (c) PS14 reaction with ADH, and (d) protein (PspA) activation with DMT-MM followed by reaction with

Fig. 1. Purification of PS14–mPspA conjugate. Elution profile of PS14–mPspA conjugate in a Phenyl Sepharose 6FF column. Equilibrium: phosphate buffer 10 mM pH 7.0 and $(\text{NH}_4)_2\text{SO}_4$ 1 M. Elution: phosphate buffer 10 mM pH 7.0 and $(\text{NH}_4)_2\text{SO}_4$ 1 to 0 M. (—) OD_{280nm}: conjugated mPspA, (–) OD_{490nm}: first peak – free unbound PS14-ADH, second peak – conjugate; (.....) OD_{280nm}: PS-ADH, and (.....) OD_{280nm}: mPspA.

PS14-ADH. Previously to the conjugation, PS14 size was reduced from about 400 kDa to about 35–60 kDa through acid hydrolysis, in order to prevent gel formation. The oxidation reaction was established to obtain about 15 moles of aldehyde per mol of PS14 and in this condition almost all aldehyde groups reacted with ADH; the residual free aldehyde groups were reduced by sodium borohydride. The conjugation was performed using PS14 and PspA in a 1:1 (mg:mg) ratio and, despite size reduction of PS14, the incubation mixture of PS14, PspA and DMT-MM resulted in gel formation. To avoid amide linkage between PspA molecules mediated by DMT-MM, PspA was previously modified by reductive methylation with formaldehyde in the presence of NaBH_3CN . Comparing the total amount of ϵ -amino groups of lysine before and after reductive methylation this reaction resulted in the modification of about 70% of the lysine residues. Using the modified PspA (mPspA), the synthesis of the PS14–mPspA conjugate was performed using PS14 and mPspA in a 1:1 (mg:mg) ratio without gel formation.

The conjugate PS14–mPspA was purified by a phenyl–sepharose chromatography eluted with a gradient from 1 to 0 M $(\text{NH}_4)_2\text{SO}_4$ (Fig. 1). In this condition, free PS14 did not bind to the phenyl–sepharose column whereas the conjugate as well as the mPspA were tightly bound. Both components were separated with water after the end of the gradient, where the conjugate is eluted first and mPspA 50 mL after the end of conjugate peak (Fig. 1, dotted line). The synthesis yield was calculated after purification and was about 20% in PS14 content and the PS14:mPspA ratio in the conjugate was about 1:2 (mg:mg). The synthesis of conjugate using different PS14:mPspA ratios (mg/mg) was attempted in order to improve the yield. However, increase of the PS14 ratio did not change the yield and the increase in mPspA ratio resulted in gel formation.

3.2. Immune response induced by modified PspA (mPspA)

In order to evaluate whether the modification of the lysine residues did not interfere in the immune response induced against PspA, sera of mice immunized with control PspA and mPspA in a 3 dose immunization scheme were compared through IgG titer by ELISA and complement deposition profile. ELISA showed induction of similar anti-PspA IgG titers (not shown) and similar complement deposition profile on three different pneumococcal strains expressing PspA family 2 clade 3 (Fig. 2).

Fig. 2. Complement C3 deposition. Complement deposition profile of antisera produced in Balb/c mice against formaldehyde modified PspA (mPspA) (---) and native PspA (...) on the pneumococcal surface of strains bearing PspA family 2, clade 3 analyzed by FACS. (A) Pneumococcal strain StP30 (PS14), (B) Pneumococcal strain P539 (PS19F), and (C) pneumococcal strain P122 (PS10A). The percentage of fluorescent bacteria (>10 fluorescence intensity units) was calculated for each sample.

3.3. Immune response induced against PspA by the conjugate PS14–mPspA vaccine

The conjugate and the controls were administered to BALB/c mice and the sera were used to evaluate the humoral immune response.

Sera of mice immunized with the conjugate contained slightly lower levels of PspA antibodies than the sera of mice immunized with co-administered antigens (PS14+mPspA) or PspA alone (Fig. 3A), suggesting that some epitopes of PspA may be hindered in the conjugate. No difference was observed in anti-PspA IgG profile between sera of mice immunized with the conjugated and co-administered PS14 plus PspA in two assays: antibody avidity and isotype distribution. The Avidity Index (AI) for the anti-PspA IgG did not change after conjugation and was calculated as 0.6 for both. Anti-PspA IgG induced against the conjugate vaccine and controls were also isotyped through quantification of IgG subclasses. No change in IgG isotype profile was observed between that induced by the conjugated and free PspA, whose quantitative distribution was: 84.3–89.4% IgG1, 5.2–6.7% IgG2a, 2.0–2.3% IgG2b and 3.3–6.6% IgG3, respectively (Fig. 3B).

In order to verify whether a lower level of antibody induction might result in lower protection, anti-PspA IgG was evaluated for its functional activity. Opsonophagocytosis is an efficient means of evaluating the induction of protective immune responses in mice and it is widely used to evaluate pneumococcal capsular polysaccharide vaccines. Since an efficient phagocytic activity is dependent on complement deposition, both assays were performed to evaluate the antisera of mice immunized with the conjugate and respective controls. Complement deposition was evaluated using a strain of pneumococcus with homologous PspA and heterologous PS, serotype 3 (PS3/PspA3). Complement deposition due to anti-PspA IgG was higher in the sera of mice immunized with the conjugate than the control co-administered (PS14+PspA), which was similar to saline, as shown in Fig. 3C. Also, in terms of the average fluorescence calculated from Fig. 3C, the fluorescence was much higher with sera of mice immunized with conjugate PS14–mPspA (40.55) than that with the co-administered antigens (PS14+mPspA), which have a value comparable to saline (12.59 and 12.03, respectively). Most importantly, the antisera of mice immunized with the conjugate reduced by 34% the survival of pneumococci containing the homologous PspA, but serotype 3 PS, strain P275/97(PS3/PspA3), in the opsonophagocytic assay, which was similar to that observed in the presence of sera from mice immunized with co-administered antigens, as shown in Fig. 3D.

3.4. Immune response induced against PS14 by the conjugate PS14–mPspA vaccine

PS14 conjugation to the mPspA protein resulted in increased induction of anti-PS14 IgG, especially after the second and third doses (Fig. 4A). Furthermore, the isotype distribution changed significantly from a profile of higher concentration of IgG3 in animals immunized with the co-administered antigens, into a profile with a higher proportion of IgG1 (~80%) in the conjugate (Fig. 4B). This increased IgG1 titer of anti-PS14 as a consequence of conjugation is a clear evidence of a well succeeded transformation of the PS14 from a thymus independent to a thymus dependent antigen [24].

High antibody avidity is usually related to increased affinity to antigen and as consequence, its efficacy in neutralizing the pathogen. Anti-PS14 IgG in sera of mice immunized with the conjugate showed an increased affinity to PS14 as demonstrated by the calculated avidity index (AI), which changed from 0.5 for anti-free PS14 IgG to 0.8 in anti-conjugated PS14 IgG. The improvement in efficacy of the PS14–mPspA vaccine is also shown by the increased C3 complement deposition in comparison to the free PS14 vaccine. Complement deposition profile was evaluated using two serotype 14 strains expressing heterologous PspA: strains 245/00 (PS14/PspA1) and P630 (PS14/PspA1), both with PspA family 1, clade 1. Complement deposition induced by sera of mice immunized with the conjugate was higher as calculated by median fluorescence units (56.13) as compared to sera from mice immunized with the co-administered antigens (38.33) for strain 245/00 (Fig. 4C) or for strain P630 (Fluorescence units, conjugate 40.55/co-administered 12.59, respectively, Fig. 4D). These results suggest that conjugation with PspA increases the protective potential of the polysaccharide moiety.

4. Discussion

Pneumococcal surface protein A (PspA) is an important virulence factor, which interferes in the binding to the mucosal bactericidal protein apolactoferrin [25] and complement deposition on pneumococci surface, reducing opsonization and clearance of bacteria by the host immune system [14]. Several vaccine formulations based on PspA have been tested with success in animal models [26–28]. For these reasons PspA could be a good candidate as protein carrier in a pneumococcal conjugate vaccine, as previously demonstrated [23,29]. PspA is expressed by all clinical isolates of *S. pneumoniae*, although it displays variability at the level of amino acid sequence. Based on the sequence variations within the B region, PspA has been classified into family 1 (clades 1 and 2), family 2 (clades 3, 4 and 5) and family 3 (clade 6) [30]. Families 1 and 2 are the most prevalent, being present in more than 90%

Fig. 3. Humoral immune response induced against mPspA. Sera of mice immunized by PS14-mPspA, and controls were tested for: (A) Anti-PspA IgG in the following groups: conjugated PS14-mPspA, and the controls, PspA, mPspA and co-administered PS14 + mPspA, (B) IgG isotype profile of the PS14-mPspA conjugate and the control PS14 + mPspA, (C) FACS analysis of complement deposition profile of the PS14-mPspA conjugate and the control PS14 + mPspA on the surface of a strain bearing PspA3 strain P275/97 (PS 3), and (D) Opsonophagocytic assay expressed as the number of CFU recovered. Pneumococcal strain P679/99 PspA3 bearing PspA3, (PS 6B) was incubated with the sera of mice immunized with the PS14-mPspA conjugate and the control PS14 + mPspA plus a complement source (NMS). Opsonized pneumococci were incubated with peritoneal macrophages, plated on blood agar plates and the surviving colonies was counted after 18 h of incubation.

of clinical isolates and therefore, PspA used in this work was from family 2, clade 3. Serotype 14 is a worldwide prevalent serotype and therefore, is an important PS to be tested in a conjugation with the protein PspA. Most of the conjugation methods need a reaction with a low activation energy intermediate molecule to allow the occurrence of the coupling of PS-ADH to protein carboxyl groups. Since the years 1980s [31], the currently used molecule to reach activation of carboxylates is EDAC. In this work, EDAC was substituted for DMT-MM. The protein carboxyls groups activation by DMT-MM occurs by a nucleophilic aromatic substitution resulting in a triazinyl ester as intermediate that reacts with nucleophiles like amine groups in protein or in polysaccharide. DMT-MM is used in carboxamide formation of small organic molecules soluble in water and alcohol [32] or in activation of carboxylates present in polysaccharides [33]. DMT-MM molecule is stable in water for at least one day [32], differently from EDAC, whose stability is dependent on pH [34] and it is easily hydrolyzed resulting in formation of side products like *N*-acylurea derivative [35]. As consequence, the reaction yield is higher with DMT-MM than with EDAC. This change allowed improvement of the reaction yield from less than 5% with EDAC (not shown) to 15–20% with DMT-MM, although this yield is still lower than that obtained with PRP from *H. influenzae* conjugated to tetanus toxoid (45%) or pneumococcal PS6B conjugated to PspA (62%) (not shown). The chemical modification of PspA through alkylation of ϵ -amino-groups of lysine by formaldehyde avoided gel formation until a concentration of 15 mg/mL of mPspA and PS14.

Modification of almost 37 lysine residues, which corresponds to 70% of the total lysine residues in the cloned PspA fragment, did not interfere in the complement deposition capacity, suggesting that most of the lysine residues are not important for the PspA protective immune response.

Although the conjugation synthesis may change the original protein epitopes profile, resulting in loss of functional properties [36,37], the method used in this work did not interfere with the induction of protective immune response. The most important function of the PspA protein in the pneumococcal infection is to prevent complement deposition. Activation of the complement system leads to deposition of complement component C3 fragments on the surface of the bacteria. Therefore, complement mediated antibody-dependent phagocytosis is also considered to be an important mechanism of pneumococcal clearance [38]. The complement deposition was higher in the sera of mice immunized with the conjugate than with the co-administered antigens, which means that the conjugation reaction improved the immune response against PspA, as had been demonstrated previously [23].

The opsonophagocytic assay (OPA), one of the assays used to evaluate plain and conjugated PS vaccines, was adapted for the PspA antigen [22] and this assay was proposed to be used instead of protection against challenge with a lethal strain. According to this assay, free and conjugated mPspA were equally capable of inducing antibodies with opsonophagocytic activity that reduces significantly the survival of pneumococci in the presence of peritoneal cells.

Fig. 4. Humoral immune response induced against PS14. Sera of mice immunized with the PS14-mPspA conjugate, and the respective controls (PS14, PS14 + mPspA), were tested for: (A) Anti-PS14 IgG, (B) IgG isotype profile induced by the PS14-mPspA conjugate or the control PS14 + mPspA antigens, and (C) FACS analysis of the complement deposition profile on the surface of two strains of pneumococci serotype 14, St245/00/PspA1 and P630/PspA1, incubated in the presence of antisera generated against the PS14-mPspA conjugate or the control PS14 + mPspA.

PspA has already been shown to be a good carrier for PS14 [29] and our results reinforce this. Furthermore, in addition to increasing the immune response to the PS, we here show that conjugation to PspA also results in an improvement in the quality of its immune response induced in terms not only of its complement deposition capacity, but also by improving the IgG avidity index and the switch in the isotype distribution profile. On a whole, our results show that a PS-mPspA conjugate can induce an efficient protective immune response against the PS and the protein moieties, broadening the protection obtained against pneumococci through either antigen and reducing the requirement for a large number of PS antigens to achieve an effective broad spectrum vaccine.

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References

- [1] Bingen E, Levy C, Varon E, De la Rocque F, Boucherat M, d'Athis P, et al. Pneumococcal meningitis in the era of pneumococcal conjugate vaccine implementation. *Eur J Clin Microbiol Infect Dis* 2008;27:191–9.
- [2] Grijalva CG, Poehling KA, Nuorti JP, Zhu Y, Martin SW, Edwards KM, et al. National impact of universal childhood immunization with pneumococcal conjugate vaccine on outpatient medical care visits in the United States. *Pediatrics* 2006;118:865–73.
- [3] Whitney CG, Farley MM, Hadler J, Hadler J, Harrison LH, Bennett NM, et al. Decline in invasive pneumococcal disease after the introduction of protein polysaccharide conjugate vaccine. *N Engl J Med* 2003;348:1737–46.
- [4] Ramani RR, Hall WN, Boulton M, Johnson DR, Zhu BP. Impact of PCV7 on invasive pneumococcal disease among children younger than 5 years: a population-based study. *Am J Public Health* 2004;94:958–9.
- [5] Dagan R, Poolman J, Siegrist CA. Glycoconjugate vaccines and immune interference: a review. *Vaccine* 2010;28:5513–23.
- [6] Knuf M, Kowalzik F, Kieninger D. Comparative effects of carrier proteins on vaccine-induced immune response. *Vaccine* 2011;29:4881–90.
- [7] Muñoz-Almagro C, Jordan I, Gene A, Latorre A, Garcia-Garcia J, Pallares R. Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. *Vaccine* 2010;28:5167–73.
- [8] Aguiar SI, Brito MJ, Gonçalo-Marques J, Melo-Cristino J, Ramirez M. Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. *Clin Infect Dis* 2008;46:174–82.
- [9] Beall B, Gherardi G, Facklam RR, Hollingshead SK. Pneumococcal PspA sequence types of prevalent multiresistant pneumococcal strains in the United States and of internationally disseminated clones. *J Clin Microbiol* 2000;38:3663–9.
- [10] Kerr AR, Paterson GK, McCluskey J, Iannelli F, Oggioni MR, Pozzi G, et al. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. *Infect Immun* 2006;74:5319–24.
- [11] Molloy S. Pneumolysin: stimulating protection. *Nat Rev Microbiol* 2011;9:4.
- [12] Gieffink C, Jelencsics KE, Gelbmann D, Senn BM, Nagy E. The pneumococcal eukaryotic type serine/threonine protein kinase StkP co-localizes with the cell division apparatus and interacts with FtsZ in vitro. *Microbiology* 2010;156:1697–707.
- [13] Castañeda E, Agudelo CI, Regueira M, Corso A, Brandileone MC, Brandão AP, et al. Laboratory-based surveillance of *Streptococcus pneumoniae* disease in children

- in 10 Latin America countries: a SIREVA II project, 2000–2005. *Pediatr Infect Dis J* 2009;28:264–70.
- [14] Andrade AL, Franco CM, Lamaro-Cardoso J, André MC, Oliveira LL, Kipnis A, et al. Non-typable *Streptococcus pneumoniae* carriage isolates genetically similar to invasive and carriage isolates expressing capsular type 14 in Brazilian infants. *J Infect* 2010;61:314–22.
 - [15] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect Immun* 2004;72:114–22.
 - [16] Silva M, Cabrera-Crespo J, Sbrogio-Almeida ME, Miyagi EM, Ho PL, Leite LCC, et al. Optimizing expression of *Streptococcus pneumoniae* surface protein A. PspA: serocross reactivity within families of antisera induced against clade 1 and 3. *Mol Biotechnol* 2007;37:146–54.
 - [17] Barazzzone CB, Carvalho Jr R, Kraschowetz S, Horta AC, Sargo CR, Silva AJ, et al. Production and purification of recombinant fragment of pneumococcal surface protein A (PspA) in *Escherichia coli*. In: *Procedia in vaccinology 4th vaccine and ISV global annual congress*. 2010.
 - [18] Cuesta G, Suarez N, Bessio MI, Ferreira F, Massaldi H. Quantitative determination of pneumococcal capsular polysaccharide serotype 14 using a modification of phenol-sulfuric acid method. *J Microb Methods* 2003;52:69–73.
 - [19] Tyllianakis PE, Kakabacos SE, Evangelatos GP, Ithakissios DS. Direct colorimetric determination of solid-supported functional groups and ligands using bichinchoninic acid. *Anal Biochem* 1994;219:335–40.
 - [20] Qi XY, Keyhani NO, Lee YC. Spectrophotometric determination of hydrazine, hydrazides, and their mixtures with trinitrobenzenesulfonic acid. *Anal Biochem* 1988;175:139–44.
 - [21] Perciani CT, Peixoto PS, Dias WO, Kubrusly FS, Tanizaki MM, Perciani CT, et al. Improved method to calculate the antibody avidity index. *Clin Lab Anal* 2007;21:201–6.
 - [22] Goulart C, Darrieux M, Rodriguez D, Pimenta FC, Brandileone MC, de Andrade AL, et al. Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. *Vaccine* 2011;29:1634–42.
 - [23] Csordas FC, Perciani CT, Darrieux M, Gonçalves VM, Cabrera-Crespo J, Takagi M, et al. Protection induced by pneumococcal surface protein A (PspA) is enhanced by conjugation to a *Streptococcus pneumoniae* capsular polysaccharide. *Vaccine* 2008;26:2925–9.
 - [24] McLay J, Leonard E, Petersen S, Shapiro D, Greenspan NS, Schreiber JR. $\gamma 3$ Gene-disrupted mice selectively deficient in the dominant IgG subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of anti polysaccharide IgG3 is corrected by induction of anti-polysaccharide IgG1. *J Immunol* 2002;168:3437–43.
 - [25] Shaper M, Hollingshead SK, Benjamin Jr WH, Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. *Infect Immun* 2004;72:5031–40.
 - [26] Tai SS. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. *Crit Rev Microbiol* 2006;32:139–53.
 - [27] Cao J, Chen D, Xu W, Chen T, Xu S, Luo J, et al. Enhanced protection against pneumococcal infection elicited by immunization with the combination of PspA, PspC, and ClpP. *Vaccine* 2007;25:4996–5005.
 - [28] Oliveira ML, Miyaji EN, Ferreira DM, Moreno AT, Ferreira PC, Lima FA, et al. Combination of pneumococcal surface protein A (PspA) with whole cell pertussis vaccine increases protection against pneumococcal challenge in mice. *PLoS One* 2010;5:e10863.
 - [29] Khan AB, Lees A, Snapper CM. Differential regulation of IgG anti-capsular polysaccharide and antiprotein responses to intact *Streptococcus pneumoniae* in the presence of cognate CD4⁺ T cell help. *J Immunol* 2004;172:532–9.
 - [30] Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* 2000;68:5889–900.
 - [31] Schneerson R, Barrera O, Sutton A, Robbins JB. Preparation, characterization, and immunogenicity of Haemophilus influenza type b polysaccharides protein conjugates. *J Exp Med* 1980;152:361–76.
 - [32] Kunishima M, Kawachi C, Hioki K, Terao K, Tani S. Formation of carboxamides by direct condensation of carboxylic acids and amines in alcohols using a new alcohol- and water-soluble condensing agent: DMT-MM. *Tetrahedron* 2001;57:1551–8.
 - [33] Schlottmann SA, Jain N, Chirmule N, Esser MT. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. *J Immunol Methods* 2006;309:75–85.
 - [34] Gilles MA, Hudson AQ, Borders Jr CL. Stability of water-soluble carbodiimides in aqueous solution. *Anal Biochem* 1990;184:244–8.
 - [35] Nakajima N, Ikada Y. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. *Bioconjug Chem* 1995;6:123–30.
 - [36] Kaboj KK, Kirchner HL, Kimmel R, Greenspan NS, Schreiber JR. Significant variation in serotype-specific immunogenicity of the seven-valent *Streptococcus pneumoniae* capsular polysaccharide-CRM197 conjugate vaccine occurs despite vigorous T cell help induced by the carrier protein. *J Infect Dis* 2003;187:1629–38.
 - [37] Leonard EG, Canaday DH, Harding CV, Schreiber JR. Antigen processing of the heptavalent pneumococcal conjugate vaccine carrier protein CRM(197) differs depending on the serotype of the attached polysaccharide. *Infect Immun* 2003;71:4186–9.
 - [38] Brown EJ, Hosea SW, Frank MM. The role of antibody and complement in thereticuloendothelial clearance of pneumococci from the bloodstream. *Rev Infect Dis* 1983;5:797–805.

Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells

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ABSTRACT

PspA is one of the most well studied pneumococcal proteins and a promising candidate for a future protein-based anti-pneumococcal vaccine. Nevertheless, its structural and serological variability suggests the inclusion of more than one PspA molecule in order to broaden protection. Since different PspAs exhibit variable levels of cross-reactivity, the selection of the protein combination with the highest coverage potential is an essential step for PspA-based vaccine development. This work investigated the level of cross-reactivity within family 1 PspAs, and established a complement based antibody mediated opsonophagocytic assay for measuring the level of cross-protection. Among a panel of ten family 1 PspA molecules, two of them, one belonging to clade 1 and another from clade 2, induced antibodies capable of enhancing complement deposition and mediating the phagocytic killing by mouse peritoneal macrophages of all pneumococci bearing PspA family 1 strains tested, regardless of their serotype. Therefore, we suggest the inclusion of either one in a PspA-based vaccine, as a representative of family 1. Furthermore, our results suggest that opsonophagocytosis by mouse peritoneal cells can be an efficient means of evaluating the induction of protective immune responses in mice across a large number of strains.

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1. Introduction

Streptococcus pneumoniae is a major cause of diseases such as meningitis, bacteremia, sinusitis, acute otitis media and pneumonia [1]. Pneumococcal diseases are responsible for millions of deaths every year, especially in developing countries [2]. The current pneumococcal vaccines are based on capsular polysaccharides. The 23-valent polysaccharide vaccine is poorly immunogenic in infants, offering clinical protection rates of about 60% in adults [3]. The 7-valent conjugate vaccine elicits protection in young children, but only against the seven included serotypes [4–7]. Recently, 10-valent and 13-valent vaccines have been licensed [8,9], but the potential replacement by non-vaccine serotypes and the high

cost reinforce the need for cost-effective strategies, such as a protein-based pneumococcal vaccine. Several proteins have been investigated as vaccine candidates against pneumococcus, including the Pneumococcal surface protein A (PspA). This is an important virulence factor, expressed on the surface of all pneumococcal strains [10], able to inhibit complement activation by the classic and alternative pathways [11]. PspA displays variability at the level of DNA sequence, although there are many sequence similarities and serologically cross-reactive epitopes [12]. The N-terminus of PspA contains the majority of protection-eliciting epitopes [13], and has been divided into three regions, A, B and C [12]. Based on the sequence variations within the B region, PspA has been classified into family 1 (clades 1 and 2), family 2 (clades 3, 4 and 5) and family 3 (clade 6) [12]. Families 1 and 2 are the most prevalent, being present in more than 90% of clinical isolates [14–17].

PspA is highly immunogenic and protective in different animal models [18]. Moreover, antibodies generated by human immunization with a single recombinant PspA showed cross-reactivity

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against PspAs from both families [19], as well as passive protection in mice challenged with *S. pneumoniae* strains bearing diverse PspAs [20].

Several studies have investigated the level of cross-reactivity among PspAs, in mice. The results suggested that the level of cross-reactivity is proportional to the degree of similarity among the amino acid sequences, with a tendency for a higher cross-reactivity within the same family [19]. Recent data indicate a considerable variation in the ability of antibodies induced against different recombinant PspAs to recognize pneumococcal isolates bearing distinct PspAs. While two family 2 fragments were found to be highly cross-reactive, the extension of cross-recognition among family 1 molecules was extremely limited; the anti-PspA1 antiserum was able to recognize all clade 1-bearing strains and half of the clade 2-containing strains tested, and the anti-PspA 2 antiserum recognized only half of the clade 2-bearing strains and two of the clade 1-expressing isolates tested [21]. The sequence analysis of *pspA* 2 has shown that the fragment used was more divergent from other clade 2 *pspA* genes sequenced by Hollingshead et al. [12]. These findings were corroborated by the limited ability of such antibodies to mediate complement deposition onto the bacterium, an important mechanism of pneumococcal clearance [22]. Altogether, these results suggest the need for selection of a more representative family 1 PspA.

The opsonophagocytic assay (OPA) has been used as a functional correlate of protection for antibodies generated against pneumococcal capsular polysaccharide. A minimum opsonic titer of 1:8 is able to confer protection in a mouse model, which correlates with protection in infants immunized with pneumococcal conjugate vaccine, corresponding to an immunoglobulin G (IgG) antibody concentration of 0.20–0.35 µg/ml [23]. However, to date, the OPA has not been well established for antibodies generated against the pneumococcal surface proteins.

Given that PspAs from the same clade can show variable degrees of cross-reactivity, the aim of this study was to determine, from a panel of Brazilian pneumococcal isolates, which is able to induce the highest level of cross-reactivity within family 1 by immunoblot, complement deposition and an opsonophagocytic assay using mouse peritoneal cells.

2. Materials and methods

2.1. Construction of PspA fragments

All cloning procedures were performed with *Escherichia coli* DH5 α grown in Luria-Bertani medium supplemented with ampicillin (100 µg/ml). DNA fragments encoding portions of the N-terminal regions of PspA clades 1 and 2 were amplified by PCR from the genomic DNA of 10 pneumococcal strains (5 of each clade). The gene products were ligated to the pGEMT-easy vector (Promega), and the sequences were confirmed by DNA sequencing. The pGEMTeasy-*pspA* constructs were digested with the appropriate restriction endonucleases and the resulting fragments were ligated to the linearized pAE-6xHis vector [24].

2.2. PspA expression and purification

Competent *E. coli* BL21(DE3) (Invitrogen) were transformed with the pAE-6xHis vectors containing the *pspA* gene fragments. Protein expression was induced in the mid-log-phase cultures by 1 mM IPTG (Sigma). The recombinant proteins, bearing an N-terminal histidine tag, were purified from the soluble fraction through affinity chromatography with Ni²⁺ charged chelating Sepharose resin (HisTrap Chelating HP; GE Healthcare) in an Akta Prime apparatus (GE Healthcare). Elution was carried out with

500 mM imidazole. The purified fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dialyzed against 10 mM Tris-HCl (pH 8) – 20 mM NaCl, and stored at –20 °C.

2.3. Pneumococcal strains

All strains used in this study are described in Table 1. Pneumococci were maintained as frozen stocks (–80 °C) in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) with 10% glycerol. In each experiment, the isolates were plated on blood agar prior to growth in THY.

2.4. Animals and immunization

Female BALB/c mice from Instituto Butantan (São Paulo, Brazil) were immunized intraperitoneally with 5 µg of recombinant PspA derivatives in saline solution 0.9% with 50 µg of Al(OH)₃ as adjuvant (500 µl per mouse). The adjuvant alone was used as a control. The animals were given three doses of protein at 7-day intervals. Sera were collected from mice at 14 and 21 days by retro-orbital bleeding. The antibody titers were examined by ELISA [21].

2.5. Analysis of serum cross-reactivity

Cross-reactivity of anti-PspA antibodies was analyzed by immunoblot. *S. pneumoniae* strains were grown in 50 ml of THY to mid- to late-log phase. Bacteria were harvested by centrifugation and the pellets were washed 3× in phosphate-buffered saline (PBS), suspended in 1 ml of 2% choline chloride (Sigma) in PBS (pH 7.0), incubated for 10 min at room temperature and centrifuged to recover the eluates [25]. Choline extracts (2 µg) from pneumococcal strains bearing PspAs of clades 1 and 2 were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Pooled anti-PspA sera (six mice per group) generated against the recombinant PspA fragments of clades 1 and 2 were added at a dilution of 1:1000 (sera collected after the second immunization), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1000; Sigma). Detection was performed with an ECL kit (GE Healthcare).

2.6. Complement deposition assay

S. pneumoniae strains (Table 1) were grown in THY to a concentration of 10⁸ CFU/ml (optical density of 0.4–0.5) and harvested by centrifugation at 2000 × g for 3 min. The pellets were washed once with PBS, resuspended in the same buffer, and incubated in the presence of heat-inactivated pooled sera from mice immunized with PspA fragments at a final concentration of 5% for 30 min at 37 °C. The sera were heat-inactivated by incubation at 56 °C for 30 min to destroy the activity of serum complement. Bacteria were then washed once with PBS, resuspended in 90 µl of gelatin Veronal buffer (Sigma), and incubated in the presence of 10% fresh-frozen normal mouse serum (from BALB/c mice) at 37 °C for 30 min. After another wash with PBS, the samples were incubated with 100 µl of FITC-conjugated goat antiserum to mouse complement C3 (MP Biomedicals) at a dilution of 1:500 on ice for 30 min in the dark. Finally, the bacteria were washed two more times with PBS, resuspended in 1% formaldehyde, and stored at 4 °C in the dark until analysis with a FACSCanto (BD Biosciences).

2.7. Opsonophagocytic assay

S. pneumoniae strains (Table 1) were grown in THY to a concentration of 10⁸ CFU/ml (optical density of 0.4–0.5) and harvested

Table 1
Pneumococcal strains used in this study.

Strain	PspA clade	Serotype	Source	Assay	Primers
M12	1	6B	UFG	Clon; IB	a; c
P13	1	9V	UFG	Clon; IB	a; c
P69	1	10A	UFG	Comp; OPA	–
P125	1	15B	UFG	Comp	–
P231	1	6A	UFG	IB	–
245/00	1	14	IAL	Clon; IB; Comp; OPA	a; d
P630	1	14	UFG	Comp	–
P1031	1	23F	UFG	Clon; IB	a; c
P1079	1	1	UFG	Clon; IB; OPA	a; c
3JY44182-95	1	3	UAB	OPA	–
M8	2	6B	UFG	IB	–
P94	2	19F	UFG	OPA	–
94/01	2	18A	IAL	Clon; IB; Comp	a; d
P278	2	18C	UFG	Clon; IB; OPA	b; d
325/95	2	6A	IAL	Clon; IB	b; d
P339	2	6A	UFG	Clon; IB	b; d
373/00	2	6B	IAL	Clon; IB; OPA	b; d
P854	2	19F	UFG	Comp; OPA	–
A66.1	2	3	UAB	IB; Comp	–
D39	2	2	UAB	IB; Comp	–

IAL: Instituto Adolfo Lutz, São Paulo, Brazil.

UFG: Universidade Federal de Goiás, Goiânia, Brazil.

UAB: University of Alabama at Birmingham, USA.

Clon: Cloning of pspA gene fragments.

IB: Immunoblot.

Comp: Complement deposition.

OPA: Opsonophagocytic assay.

a: Forward 5'-GAAGCGCCGTAGCTSGTC-3'.

b: Forward 5'-ACCATGGTAAGAGCAGAAGAAGCC-3'.

c: Reverse 5'-TTATTCTGGTTTAGGAGCTGGAGCTGG-3'.

d: Reverse 5'-CCACATACCGTTTCTGTTCCAGCC-3' [12].

by centrifugation at $2000 \times g$ for 3 min. The pellets were washed once with PBS, resuspended in the opsono buffer [26], and aliquots containing 2.5×10^6 CFU were incubated with heat-inactivated anti-PspA 94/01 or 245/00 pooled sera at a final dilution of 1:8 and 1:16 at 37°C for 30 min. Sera from mice immunized with Alum were used as control. After another wash with PBS, the samples were incubated with 10% normal mouse sera (NMS) diluted in opsono buffer at 37°C for 30 min. The samples were then washed once with PBS and incubated with 4×10^5 peritoneal cells (see Section 2.8) diluted in opsono buffer, at 37°C for 30 min with shaking (220 rpm). The reaction was stopped by incubation on ice for 1 min. Ten fold dilutions of the samples were performed and $10 \mu\text{l}$ aliquots of each dilution were plated on blood agar plates. The plates were incubated at a 37°C , 5% CO_2 and the pneumococcal CFU recovered counted after 18 h. The slides were prepared by cyto spin and stained with Instant-Prov (Newprov, Brazil). Statistical analysis of the final pneumococcal counts in each group was performed by one-way ANOVA with a Tukey's Multiple Comparison Test.

2.8. Peritoneal cells

BALB/c mice were injected i.p. with $10 \mu\text{g}$ of Concanavalin A from *Canavalia ensiformis* (ConA, Sigma), sacrificed 48 h after treatment and their peritoneal cavities washed with 5 ml of ice-cold PBS. The peritoneal cells were adjusted to $4 \times 10^6/\text{ml}$ in opsono buffer [27].

3. Results

3.1. PspA expression and purification

The N-terminal regions of 10 family 1 PspAs (5 clade 1 and 5 clade 2) from Brazilian pneumococcal strains (Table 1) were expressed in fusion with a His-tag in competent *E. coli* strains and purified through Ni^{2+} affinity chromatography. The SDS-PAGE of

Fig. 1. SDS PAGE of the purified recombinant PspAs ($5 \mu\text{g}$). The N-terminal region of ten family 1 PspAs was expressed in *E. coli* strains in fusion with His-tag and purified by Ni^{2+} affinity chromatography. Molecular mass markers (kDa) are indicated on the left.

the purified recombinant proteins shows that the molecular mass varied from ~ 45 to 70 kDa (Fig. 1). All fragments included portions of the proline-rich region, and PspAs 245/00, P1031, 325/95, P339 and 94/01 also comprised the non-proline block.

3.2. Cross-reactivity

Polyclonal sera from BALB/c mice immunized with two or three doses of recombinant PspAs were examined by ELISA and showed similar antibody titers (data not show). The pooled anti-PspA antisera were tested for their ability to recognize several choline extracts from pneumococcal strains bearing PspAs of clades 1 (six strains) and 2 (seven strains) by immunoblot. The results are shown in Fig. 2. The analysis of serum cross-reactivity among PspAs from clades 1 and 2 revealed a significant variation in the level of recognition of different isolates. Of all antisera tested, four presented high levels of cross-reactivity with PspAs of both clades, being two from clade 1 – PspA M12 and 245/00 – and two from clade 2 – PspA

Fig. 2. Analysis of serum cross-reactivity within PspA family 1 by immunoblot. Polyclonal sera from mice immunized with the N-terminal regions of PspAs from 10 family 1 pneumococci (5 of each clade) were tested for their ability to recognize diverse choline extracts (2 μ g) of pneumococcal strains bearing PspAs clade 1 and 2. Molecular mass markers (kDa) are indicated on the left. The positive controls are underlined on the top right of each blotting. The four most cross-reactive anti-sera are highlighted.

94/01 and P339. These sera were selected and tested for their ability to increase complement deposition on the surface of a panel of pneumococcal stains.

3.3. Complement deposition in the presence of anti-PspA antibodies

We also determined the ability of the four selected anti-PspA sera to increase complement deposition on the surface of various pneumococci. Eight pneumococcal strains expressing family 1 PspAs were incubated with the heat-inactivated pooled sera from: PspA 245/00, PspA M12, PspA 94/01, PspA P339, PspA P 278 or serum from mice injected with only Al(OH)₃ followed by the addition of 10% fresh-frozen normal mouse serum. The samples were washed and labeled with FITC-conjugated goat anti-mouse C3. The percentage of bacteria coated with C3 >10 fluorescence intensity units was determined by flow cytometry.

Antibodies generated against PspA 245/00, when incubated with pneumococcal strains expressing clade 1 PspAs, efficiently increased C3 deposition, in all serotypes tested. Interestingly, the same was observed with strains bearing clade 2 PspAs, even strain A66.1, which is a heavily encapsulated serotype 3 strain (Figs. 3 and 4). Fig. 4 summarizes the complement deposition results, after discounting the non-specific interaction, revealing a percentage of fluorescent bacteria not lower than 30% for all strains tested. On the other hand, antibodies generated against PspA M12 induced lower C3 deposition in both PspA clade 1 and clade 2 containing strains (Figs. 3 and 4). As for antibodies produced against

PspA clade 2, anti-PspA 94/01 enhanced the amount of C3 deposited on all bacteria tested, regardless of the PspA clade expressed on their surface. Anti-PspA P339, on the other hand, showed the poorest results, leading to an increase in the amount of C3 deposited on only half of the pneumococcal strains tested. Corroborating with the immunoblot results, a poorly cross-reactive serum in that assay, P278, also showed a reduced ability to induce complement deposition in most of the strains (Figs. 3 and 4).

In summary, antibodies generated against PspA 245/00 and 94/01 were able to increase complement deposition on the widest range of pneumococci tested, being selected for further investigation of their potential to mediate opsonophagocytic killing by peritoneal cells.

3.4. Opsonophagocytic assay

The two most cross-reactive sera selected from complement deposition assays, anti-PspA 245/00 and 94/01, were tested for their ability to block PspA's anti-phagocytic activity, therefore promoting pneumococcal opsonization by deposition of complement and phagocytosis by peritoneal cells (macrophages and neutrophils). The antisera were tested at two different dilutions, 1:8 and 1:16. Fig. 5 shows the number of CFUs recovered after incubation of pneumococci with peritoneal cells in the presence of sera at the dilution of 1:16 with the exception for Strain P 1079 in which the anti-PspA 94/01 opsonophagocytic activity was observed only at a dilution of 1:8. The anti-PspA 245/00 antisera (clade 1) was able to reduce the number of CFUs recovered in at least 40% for strains

Fig. 3. Comparison of complement protein C3 deposition onto pneumococcal surface in the presence of the selected anti-PspA sera. Strains bearing PspAs clade 1 (P69, P125, 245/00 and P630), and clade 2 (A66.1, D39, 94/01 and P854) were incubated with sera from mice immunized with recombinant PspAs. (Anti-PspA M12, Anti-PspA 245/00, Anti-PspA P 339, Anti-PspA 94/01 and Anti-PspA P 278). Serum from mice immunized with Alum was used as a control for each bacterium and is represented by the gray-shaded areas. The percentage of fluorescent bacteria (>10 fluorescence intensity units) was calculated for each sample.

bearing PspA clade 1 and 30% for strains containing clade 2 PspA, reaching a maximum of 50% in strains of the same clade. Furthermore, sera from mice immunized with PspA 94/01 (clade 2), was able to mediate killing of at least 30% of the bacteria expressing PspAs clade 1 or 2. The only exception was that of strain P278, for which the reduction in CFU recovered was only 17%. The maximum reduction induced by anti-PspA 94/01 antisera was 46 and 63% for strains bearing PspA 1 and 2, respectively. The CFU reduction mediated by anti-PspA 245/00 and 94/01 was statistically significant when compared to serum from mice receiving Aluminum hydroxide (except for strain P 278). Both sera induced similar degrees of bacterial phagocytosis among pneumococci bearing family 1 PspAs, since there were no statistically significant differences between the effect induced by anti-PspA 245/00 and anti-PspA 94/01 antisera.

Microscopical analysis of the samples revealed the interaction between the phagocytes and the pneumococci incubated with both

sera (Fig. 6). In the control group, after incubation of the cells with bacteria previously treated with non-specific antibodies, no interaction was observed, as depicted by the mononuclear cell in Fig. 6A. On the other hand, incubation of the cells with a PspA clade 1 expressing strain, previously opsonized with anti-PspA 94/01 (clade 2), induced a strong interaction between the bacteria and the peritoneal cells, as demonstrated by the pneumococci-covered macrophage in Fig. 6B. Noteworthy is the ability of the anti-PspA 94/01 antibodies to mediate phagocytosis of a pneumococcal strain expressing a heterologous PspA, a strong indication of cross-protection. A similar result was obtained when cells were cultured in the presence of the pneumococcal strain P 69, containing PspA clade 1, previously incubated with anti-PspA245/00, also clade 1; Fig. 6C and D shows a large number of internalized bacteria in a macrophage and a neutrophil, respectively.

Fig. 4. Complement deposition onto pneumococcal surface in the presence of Anti-PspA. Percentages of bacteria positive for C3 deposition after incubation with antisera are shown. The values of bacteria incubated with serum from mice receiving Alum were discounted.

4. Discussion

PspA is a promising vaccine candidate against pneumococcal disease; however, its structural and serological variability could limit the coverage of a PspA-based vaccine. Therefore, understanding the nature of PspA's variability has been the focus of many studies regarding anti-pneumococcal vaccine development. Hollingshead et al. [12], grouped most PspAs into two major families, 1 and 2, which were subdivided into 5 clades. PspAs of the same family share 55–80% of the so-called clade defining region, while sequence similarity between families is <55%. It has been demonstrated that the level of cross-reactivity and cross-protection among PspAs correlates with sequence similarity, being low between PspAs of different families and higher within each family. Furthermore, it has been suggested that the level of cross-reactivity and cross-protection varies depending on the PspA clade [21]. In that study, a PspA from clade 3 elicited antibodies with the lowest cross-reaction, while PspAs 4 and 5 (belonging to family 2) were highly cross-reactive. For family 1 molecules, neither PspA clade 1 nor clade 2 were able to induce antibodies cross-reactive to all family 1 strains tested. Therefore, further research was needed to better understand cross-reactivity within family 1.

In the present study, the N-terminal regions of five clade 1 and five clade 2 PspAs were produced, antibodies generated and screened for their cross-reactivity against a panel of Brazilian strains containing clade 1 and 2 PspAs. The immunoblot analysis revealed a high heterogeneity in the level of cross-reactivity of the different antisera; while most cross-reacted mainly within the homologous clade, four PspAs – 245/00, M12, 94/01 and P339 – generated antibodies able to recognize most of the isolates tested. There was no predominance between the PspA clade and the level of cross-reaction; clades 1 and 2 were equally cross-reactive.

The hybridization of the reverse primers in distinct regions within the proline-rich moiety generated fragments with different sizes; all fragments included the entire α -helical domain plus the beginning of the proline-rich region, and some were longer, containing most of the proline block, several including the nonproline block. Although there was no clear correlation between the size of the fragment and the level of cross-reactivity by immunoblot – the most cross-reactive fragments included both long and short

proteins – in the more stringent assays – complement deposition and OPA – the two best candidates included the proline-rich region with the nonproline block. This result suggests a possible role for the proline-rich region with the nonproline block in the induction of functional antibodies. This data is in agreement with a recent study demonstrating that immunization of mice with the proline-rich region including the nonproline block was able to protect mice against fatal challenge [28].

Complement mediated antibody-dependent phagocytosis is considered to be an important mechanism of pneumococcal clearance [29]. The ability of anti-PspA antibodies to promote complement deposition on the bacterial surface greatly contributes to their protective effect [11]. It has been demonstrated, however, that the level of complement deposited depends on the similarity between the PspA used to induce the antibodies and that expressed by the pneumococcus [21,30]. In order to assess this important functional property, antibodies to the four selected PspAs were tested for their ability to induce complement deposition on several pneumococcal strains. Flow cytometric analysis of the interaction of the generated antibodies with diverse pneumococci showed that antibodies to PspA 245/00 and 94/01 were able to increase complement deposition on the widest range of pneumococci tested. The complement deposition on the different pneumococci appeared to be also influenced by the serotype. We observed that some serotypes exhibited an increased complement deposition in the absence of anti-PspA antibodies, as demonstrated previously with serotype 6B strains [31].

We tested the ability of these antisera to induce the complement deposition in pneumococcal strains bearing family 2 PspAs (data not shown), and no increase in complement deposition was observed. This result is in accordance with our previous findings [21], and suggests that, although some family 1 molecules can broaden cross-reactivity within this family, this effect is not extended to family 2. Our results demonstrated a significant variability in the cross-reactivity of antisera generated against PspAs of the same clade, which correlates with differences in antibody mediated complement deposition on pneumococci.

In order to correlate the results of cross-reactivity with protection, we evaluated the ability of the two most cross-reacting sera to promote the opsonophagocytosis of different pneumococ-

Fig. 5. Opsonophagocytic assay: Pneumococcal strains bearing family 1 PspAs were incubated with serum from mice immunized with recombinant PspAs 245/00 or 94/01 (clade 1 and 2, respectively) and NMS (complement source). The opsonized pneumococci were incubated with peritoneal cells and plated on blood agar plates. Serum from mice immunized with Alum was used as a control for each bacterium. The number of CFU recovered after 18 h were compared by one-way ANOVA with a Tukey's Multiple Comparison Test. The bars represent the standard error of the mean (SEM) and the asterisks indicate statistically significant differences (** $p < 0.001$; * $p < 0.01$). Results are representative of two independent experiments.

Fig. 6. Phagocytosis of *S. pneumoniae* by murine peritoneal cells: (A) negative control, peritoneal macrophages incubated with the pneumococcal strain 491/01 pre-opsonized with serum from mice immunized with Alum; (B) peritoneal macrophages after phagocytosis of strain 491/01 opsonized with anti-PspA 94/01; (C) peritoneal macrophages after phagocytosis of strain P69 opsonized with anti-PspA 245/00; (D) peritoneal neutrophil after phagocytosis of strain 245/00 opsonized with anti-PspA 245/00.

cal strains by peritoneal phagocytes. Since it has been difficult to show killing using the classical OPA by anti-PspA antisera (unpublished data), we have optimized this assay in order to overcome the protective effect of the capsule. Using peritoneal cells recovered from mice stimulated with a polyclonal T-cell activator, we were able to demonstrate the ability of anti-PspA antibodies to induce complement mediated phagocytosis of pneumococci of different serotypes. The results demonstrate that both sera were able to induce complement-mediated phagocytosis leading to a minimum reduction of 30% on the number of pneumococci. This effect was observed for pneumococci of diverse capsular types, including serotypes 1, 3 and 6B, demonstrating the viability of this adapted opsonophagocytic assay for measuring the protective role of anti-PspA antibodies, which can overcome the inhibitory effects of different capsule types. Although these two sera were generated against PspAs of different clades, both were equally efficient against all family 1 strains. These results are in accordance with the complement deposition assay, in which both sera were able to increase complement deposition onto pneumococci containing PspA clades 1 and 2. This cross-reactive effect within strains bearing family 1 PspA has been previously reported using anti-PspA1 antibodies [21,22]. Moreno et al. [22] also demonstrated that the immunization of mice with PspA 4 or PspA 5 was able to induce cross-protection against intranasal challenge with a PspA clade 2 bearing strain. More recently, immunization with a clade 5 PspA using DTP as an adjuvant was able to broaden cross-protection against family 1 strains, in an intranasal challenge model [32].

Altogether, our results indicate that antibodies generated against PspAs of the same clade induce different levels of cross-reactivity. The sera induced against two PspAs 245/00 and 94/01, clade 1 and clade 2, respectively, were able to induce greater complement deposition on pneumococcal strains containing PspAs from family 1. Furthermore, these two sera were able to induce the opsonophagocytosis of pneumococcal strains by peritoneal cells reducing CFU recovery, suggesting a potential protective effect. We therefore suggest that the inclusion of either one of the two PspAs, 245/00 or 94/01, in a PspA-based anti-pneumococcal vaccine could induce broad protection against pneumococcal strains containing family 1 PspAs. This protein could be used in combination with a family 2 molecule, selected by a similar strategy, in order to extend protection to pneumococcal strains bearing PspAs of both families 1 and 2, which should provide a high coverage.

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References

- [1] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 2009;374(September (9693)):893–902.
- [2] Pneumococcal conjugate vaccine for childhood immunization – WHO position paper. *Wkly Epidemiol Rec* 2007;82(March (12)):93–104.
- [3] Bogaert D, Hermans PW, Adrian PV, Rumke HC, de Groot R. Pneumococcal vaccines: an update on current strategies. *Vaccine* 2004;22(June (17–18)):2209–20.
- [4] Hansen J, Black S, Shinefield H, Cherian T, Benson J, Fireman B, et al. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than 5 years of age for prevention of pneumonia: updated analysis using World Health Organization standardized interpretation of chest radiographs. *Pediatr Infect Dis J* 2006 Sep;25(9):779–81.
- [5] Ghaffar F, Barton T, Lozano J, Muniz LS, Hicks P, Gan V, et al. Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. *Clin Infect Dis* 2004;39(October (7)):930–8.
- [6] Brandileone MC, de Andrade AL, Di Fabio JL, Guerra ML, Austrian R. Appropriateness of a pneumococcal conjugate vaccine in Brazil: potential impact of age and clinical diagnosis, with emphasis on meningitis. *J Infect Dis* 2003;187(April (8)):1206–12.
- [7] Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, Craig AS, et al. Incidence of pneumococcal disease due to Non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the Era of widespread PCV7 vaccination, 1998–2004. *J Infect Dis* 2007;196(November (9)):1346–54.
- [8] Licensure of a 13-valent pneumococcal conjugate vaccine (PCV13) and recommendations for use among children – Advisory Committee on Immunization Practices (ACIP). *MMWR Morb Mortal Wkly Rep* 2010;59(March (9)):258–61.
- [9] Vesikari T, Wysocki J, Chevallier B, Karvonen A, Czajka H, Arsene JP, et al. Immunogenicity of the 10-valent pneumococcal non-typeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine. *Pediatr Infect Dis J* 2009;28(April (4 Suppl.)):S66–76.
- [10] Yother J, Handsome GL, Briles DE. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the pspA gene. *J Bacteriol* 1992;174(January (2)):610–8.
- [11] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect Immun* 2004;72(January (1)):114–22.
- [12] Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* 2000;68(October (10)):5889–900.
- [13] McDaniel LS, Ralph BA, McDaniel DO, Briles DE. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. *Microb Pathog* 1994;17(November (50)):323–37.
- [14] Baril L, Briles DE, Crozier P, King J, Punar M, Hollingshead SK, et al. Characterization of antibodies to PspA and PsaA in adults over 50 years of age with invasive pneumococcal disease. *Vaccine* 2004;22(December (6)):789–93.
- [15] Hollingshead SK, Baril L, Ferro S, King J, Coan P, Briles DE. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. *J Med Microbiol* 2006;55(February (Pt 2)):215–21.
- [16] Pimenta FC, Ribeiro-Dias F, Brandileone MC, Miyaji EN, Leite LC, Sgambatti de Andrade AL. Genetic diversity of PspA types among nasopharyngeal isolates collected during an ongoing surveillance study of children in Brazil. *J Clin Microbiol* 2006;44(August (8)):2838–43.
- [17] Brandileone MC, Andrade AL, Teles EM, Zanella RC, Yara TI, Di Fabio JL, et al. Typing of pneumococcal surface protein A (PspA) in *Streptococcus pneumoniae* isolated during epidemiological surveillance in Brazil: towards novel pneumococcal protein vaccines. *Vaccine* 2004;22(September (29–30)):3890–6.
- [18] Tai SS. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. *Crit Rev Microbiol* 2006;32(3):139–53.
- [19] Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine* 2000;18(March (17)):1743–54.
- [20] Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J Infect Dis* 2000 Dec;182(6):1694–701.
- [21] Darrieux M, Moreno AT, Ferreira DM, Pimenta FC, de Andrade AL, Lopes AP, et al. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *J Med Microbiol* 2008;57(March (Pt 3)):273–8.

- [22] Moreno AT, Oliveira ML, Ferreira DM, Ho PL, Darrieux M, Leite LC, et al. Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. *Clin Vaccine Immunol* 2010;17(March (3)): 439–46.
- [23] Jodar L, Butler J, Carlone G, Dagan R, Goldblatt D, Kayhty H, et al. Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. *Vaccine* 2003;21(July (23)):3265–72.
- [24] Ramos CR, Abreu PA, Nascimento AL, Ho PL. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 2004;37(August (8)):1103–9.
- [25] Briles DE, King JD, Gray MA, McDaniel LS, Swiatlo E, Benton KA. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. *Vaccine* 1996;14(June (9)):858–67.
- [26] Romero-Steiner S, Libutti D, Keyserling HL, Carlone GM. *Streptococcus pneumoniae* opsonophagocytosis using differentiated HL-60 cells Center for Disease Control and Prevention and Emory University Atlanta, GA; 1999.
- [27] Rodrigues D, Cavada BSA-D-O JT, Moreira RA, Russo M. Differences in Macrophagesstimulation and leukocyte Accumulation in response to Intraperitoneal administration of Glucose/Mannose-Binding Plant Lectins. *Braz J Med Biol Res* 1992;25:823–6.
- [28] Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, et al. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. *Infect Immun* 2010;(March).
- [29] Brown EJ, Hosea SW, Frank MM. The role of antibody and complement in the reticuloendothelial clearance of pneumococci from the bloodstream. *Rev Infect Dis* 1983;5(September–October (Suppl. 4)):S797–805.
- [30] Xin W, Li Y, Mo H, Roland KL, Curtiss 3rd R. PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhimurium extend and enhance protection against *Streptococcus pneumoniae*. *Infect Immun* 2009;77(October (10)):4518–28.
- [31] Melin M, Jarva H, Siira L, Meri S, Kayhty H, Vakevainen M. *Streptococcus pneumoniae* capsular serotype 19F is more resistant to C3 deposition and less sensitive to opsonophagocytosis than serotype 6B. *Infect Immun* 2009;77(February (2)):676–84.
- [32] Oliveira ML, Miyaji EN, Ferreira DM, Moreno AT, Ferreira PC, Lima FA, et al. Combination of pneumococcal surface protein A (PspA) with whole cell pertussis vaccine increases protection against pneumococcal challenge in mice. *PLoS One* 2010;5(5):e10863.

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Production and purification of recombinant fragment of pneumococcal surface protein A (PspA) in *Escherichia coli*

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Abstract

New conjugated vaccines against *Streptococcus pneumoniae* are being developed using pneumococcal surface proteins as carriers. The pneumococcal surface protein A (PspA) was selected as carrier because it is indispensable for virulence of *S. pneumoniae*. The PspA can be classified into 3 families according to the homology of protein sequences, within each family there is immunological cross-reactivity and PspA from family 1 or 2 are present in 99% of strains associated with pneumococcal invasive disease. Hence, the purpose of this work was to develop an industrial production and purification process of His-tagged recombinant fragment of PspA in *E. coli* BL21 (DE3), rFPspA245 from family 1.

Fed-batch cultivations in 5-L bioreactors with defined medium were carried out using glycerol as carbon source. It was obtained circa 60 g/L of dry cell weight and 3.0 g/L of rFPspA. Cells were disrupted with 96.7% of efficiency by high pressure continuous homogenizer. The clarification step was done by centrifugation. The results of chromatographic steps were analyzed by densitometry of SDS-PAGE protein bands. Using the chromatographic sequence anion exchange (Q-Sepharose) followed by metal affinity (IMAC-Sepharose), the rFPspA245 was obtained with 67% and 97% of purity respectively for each step and final recovery of 23%. In conclusion, the purification process was developed and rFPspA245 was obtained with high purity, but the recovery should still be improved.

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1. Introduction

Streptococcus pneumoniae is a Gram-positive microorganism with a polysaccharide capsule, causative agent of bacterial pneumonia, otitis media, meningitis and sinusitis. It is a human pathogen transmitted from person to person by aerosol.

The incidence of pneumococcal disease and mortality is higher in children under 5 years of age, in the elderly and in immunocompromised individuals [1, 2]. A mortality rate of 5 - 22% has been associated with the initial phase of the disease and even in cases with antibiotic susceptible strains; there is still a mortality of 10% in pneumonia and 30% in meningitis [3], more frequent in the first months of life [4]. This situation is worse when chemotherapy fails due to resistant strains [5].

The most important pneumococcal virulence factor is the capsular polysaccharide, whose structure defines the serotypes. In Brazil, there are 12 prevalent serotypes, which are responsible for 75% of the pneumococcal infections, and 45% of the pneumococcal infections are related to the serotypes 14, 6B and 1. The serotypes 1 and 6B are prevalent in all ages; the serotype 14 is prevalent in children and the 3 and 4 in adults [4].

Nomenclature

PspA	Pneumococcal surface protein A
IMAC	immobilized metal-ion affinity chromatography
kan	kanamycin
HCD	high cell density
OD	optical density
HPLC	high performance liquid chromatography
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
$m_s(t)$	glycerol mass flow rate (g/h)
$Y_{X/S}$	yield factor on biomass (g dry cell weight/g consumed glycerol)
μ_{set}	desired specific growth rate during the fed-batch phase (h^{-1})
m	maintenance coefficient (0.025 g/g.h)
X_F	cell concentration at the beginning of feeding (g dry cell weight/L)
V_F	medium volume (L) at the beginning of feeding
t_F	instant at the beginning of feeding (h)
t	time
IPTG	isopropyl β -D-thiogalactopyranoside
PMSF	phenylmethylsulfonyl fluoride
kDa	kilo Daltons

So far all the current pneumococcal available vaccines are based on free capsular polysaccharides or polysaccharides conjugated to carrier proteins. Conjugation of the polysaccharide with carrier proteins induces T-cell dependant immunity, high antibody production and memory B-cells. The first used conjugated pneumococcal vaccine was licensed in 2000 (Prevenar, Wyeth) containing 7 serotypes (4, 6B, 9V, 14, 18C, 19F e 23F) conjugated with a mutated diphtheria toxin, CRM197. This formulation has been effective against the pneumococcal invasive disease in children lower than 2 years old [6]. The more commonly used carrier proteins are the tetanus and diphtheria toxoids [7], the genetically mutated diphtheria toxin CRM₁₉₇ [8] and the outer membrane protein complex of *Neisseria meningitidis* [9]. Although the conjugated pneumococcal vaccines are highly considered in the invasive infection, the variability of serotypes continues to be an obstacle [10], technically limiting the number of possible antigens included in the vaccine [11].

In order to broaden the coverage of the vaccine, a new conjugate vaccine using the three most prevalent polysaccharides in Brazil, serotypes 14, 6B and 1, conjugated to PspA are being developed. The PspA was shown to induce systemic antibody response and protection against challenge with a virulent strain in mice by a mechanism of inhibition of complement deposition in the bacterial surface. Furthermore, since the PspA structure is like a long tail, there is a region which is not covered by the capsule [12]. The N-terminal region of PspA contains most of the immunogenic epitopes of this molecule [13] and is capable of protecting mice against an invasive challenge with virulent pneumococci [14]. However, this region exhibits serological variability, leading to the classification of the protein in three families. The family 1 consists of clades 1 and 2, family 2 of clades 3, 4 and 5, and family 3 of clade 6 [15]. The families 1 and 2 are present in around 99% of pneumococcal strains; therefore the N-terminal region of one representative of family 1, PspA245 was chosen to be cloned in *Escherichia coli* in order to develop the production process for the carrier proteins of this new conjugate pneumococcal vaccine.

E. coli is the bacterium most used in expression of heterologous protein. This system allows obtaining high-density cell cultures [16]. However, rarely, the recombinant protein is obtained in the broth culture. So, it is necessary to lyse the cells to extract the protein of interest. Due the enormous amount of impurities released by the lysis, several purification steps are necessary. After lysis, clarification and chromatographic steps are employed. The right choice of chromatographic conditions can generate the protein in high yield and purity degree [17]. Ion exchange chromatography is widely used because is simple to operate, allows a greater flow and has a lower cost. Despite of higher cost, immobilized metal-ion affinity chromatography (IMAC) is also very common due to the fact His-tagged recombinant proteins, as the fragments of PspA which were synthesized with six histidine residues, have affinity for metals like Ni⁺² [18].

2. Materials and Methods

2.1. Production of rfpPspA245 in *E. coli* BL21(DE3)

The N-terminal fragment of pspA245 gene was cloned into pET37b+ and expressed in *E. coli* BL21(DE3). The frozen stock was spread in agar M9 medium with kanamycin (kan). High cell density (HCD) medium [19] containing 20 g/L glycerol was used for cultivation of the inoculum and the same medium with 40 g/L glycerol was used for batch cultures in 5L-reactor BioFlo 2000 (New Brunswick). The cell concentration was measured by OD_{600nm}. The glycerol and acids concentrations were analyzed by HPLC (Aminex HPX-87H, BioRad) and the protein by SDS-PAGE 12%. The mass flow rate of the

carbon source during the fed-batch phase was calculated according to the equation (1) and the induction was done with 1 mM IPTG.

$$m_S(t) = \left[\frac{1}{Y_{X/S}} \cdot \mu_{set} + m \right] \cdot X_F \cdot V_F \cdot e^{\mu_{set} \cdot (t - t_F)} \quad (1)$$

2.2. Purification of rfPspA245 produced in *E. coli* BL21(DE3)

After the cultivation, the cellular suspension was centrifuged (17,969 g) by 30 minutes at 4°C. The cell mass was frozen. The cell pellets (400g) were resuspended in 1.0 L of lysis buffer (25 mM tris pH 8.0 + 0.1% triton X-100) with a protease inhibitor, 1.0 mM PMSF. A homogeneous suspension was obtained in a mixer (CAT X520) and disrupted by a high pressure continuous homogenizer (APV-Gaulin) in a close loop for 12 minutes at 600 bar. The homogenizer has a jacketed reservoir and a tube-and-shell heat exchanger in the inlet and outlet, respectively, to control the temperature during the lysis under 12°C. Samples were taken every minute to determine the efficiency of lysis. The 100% of lysis efficiency was considered the OD600nm of the cell suspension after treatment with 0.1M NaOH.

The homogenate clarification was done by centrifugation (17,696 g) for 2 h at 4°C. The supernatant was filtrate in a membrane (0.45 µm) to obtain the clarified homogenate.

The chromatographic steps were done using an Äkta Explorer (GE Healthcare). The flow was 50 mL/min in columns XK 50. The resins employed were Q-Sepharose Fast Flow (anion exchange) and IMAC-Sepharose (immobilized metal-ion affinity chromatography). All material was purchased from GE Healthcare.

We evaluated two chromatographic sequences: Q-Sepharose followed by IMAC-Sepharose and IMAC-Sepharose followed by Q-Sepharose. In the case of Q-Sepharose, the elution buffer was 25 mM sodium acetate pH 6.5 + 200 mM NaCl. IMAC-Sepharose was loaded with NiSO₄ and the elution was done with 20 mM phosphate buffer pH 7.4 + 200 mM imidazol. The conditions of binding, wash, elution and cleaning are showed in Figure 1 (Q-Sepharose) and Figure 2 (IMAC-Sepharose).

Fig. 1. Chromatographic conditions used for purification of rfPspA245 in Q-Sepharose.

IMAC - Sepharose

Fig. 2. Chromatographic conditions used for purification of rfPspA245 in IMAC-Sepharose.

2.3. Analytical Methods

Protein quantification was done according to Bradford [20], using Bradford reagent from Sigma-Aldrich. SDS-PAGE was carried out under reducing conditions in a 12% gel according to Laemmli et al. [21]. The relative purity, considered as the percentage of the intensity of PspA245 band against the sum of intensity of all other bands in the lane, was determinate by densitometry of SDS-PAGE protein bands in a Biorad GS-800 densitometer and analyzed by Quantity One 4.6.3 software.

3. Results and Discussion

The rfPspA245 was produced using glycerol, a by-product of the Brazilian biofuel industry. It was obtained circa 60 g/L of dry cell weight and 3.0 g/L of rfPspA. Using glycerol as carbon source, the acetate formation was lower than 1.0 g/L during all process (not shown). The biomass production was similar to that previously obtained using glucose as carbon source (not shown).

The Figure 3 shows the rfPspA245 production in high cell density of *E. coli* using glycerol as carbon source.

Fig. 3. Production of rfPspA245 in fed-batch cultures using glycerol as carbon source. Lane 1: before induction; lanes 2-5: 1-4 h of induction, respectively.

The cell disruption was successfully achieved using a mechanical (high pressure continuous homogenizer) and chemical (detergent Triton X-100) combined method, reaching 96.7% of efficiency.

The first chromatographic sequence, Q-Sepharose followed by IMAC-Sepharose, was used to the purification rfPspA245. The conditions were described above (Figures 1 and 2). The electrophoresis gels of purification are shown in Figure 4 and 5. The results are described in Table 1.

Considering the results described in Figure 4, 5 and Table 1, we can verify that the protein is not present in fraction QF1 but in fraction QF2 is present with higher purity, 44.8%, than in loading fraction 34.9%. In the trade off, the purity was selected instead of recovery and the QF3 fraction was obtained with a purity of 88.1%.

Fig. 4. SDS-PAGE of rfPspA245 purification in Q-Sepharose. Lane 1: molecular marker (kDa); lane 2: clarified homogenate; lane 3: Q loading fraction; lane 4: QF1, flow-through; lane 5: QF2, wash 1; lane 6: QF3, elution; lane 7: QF4, wash 2; lane 8: QF5, cleaning.

Fig. 5. SDS-PAGE of rfPspA245 purification in IMAC-Sepharose. Lane 1: IMAC loading fraction; lane 2: NiF1, flow-through; lane 3: NiF2, wash 1; lane 4: NiF3, wash 2; lane 5: NiF4, elution; lane 6: molecular marker (kDa).

Table 1. Purification of rfPspA245 in Q-Sepharose followed by IMAC-Sepharose

Sample	Total Protein (mg)	Relative Purity rfPspA245 (%) [#]	rfPspA245 (mg)	Recovery rfPspA245 (%)	Purification Factor
Clarified Homogenate	62010	34.9	21641	100.0	1.0
Q-Sepharose (QF3)	10120	88.1	8916	41.2	2.5
IMAC –Sepharose (NiF4)	5200	96.6	5023	23.0	2.8 (1.1)*

[#] Calculated by densitometry. * Value between parentheses is the purification factor of this step

In the first chromatographic sequence tested for purification of PspA245, we obtained the necessary purity degree after the IMAC-Sepharose (96.9%). However, the recovery of PspA245 was low (23%). Analyzing the SDS-PAGE (Figure 5) we can observe PspA245 in all fractions of IMAC-Sepharose. So, the chromatographic conditions could be changed in order to increase the recovery.

The second chromatographic sequence tested for purification of PspA245 consisted of IMAC-Sepharose followed by Q-Sepharose. The conditions are the same described in Figures 1 and 2. The Figures 6 and 7 and Table 2 show the results. This chromatographic sequence was not indicated to the purification of rfPspA245. The relative purity (79.9%) and the final recovery (9.1%) were lower than using the inverse sequence.

Besides the better results obtained with the first chromatographic sequence (Q-Sepharose followed by IMAC-Sepharose), the use of Q-Sepharose as the first chromatographic step has the advantage of increasing the life-time of the most expensive resin, IMAC-Sepharose.

Fig. 6. SDS-PAGE of rfPspA245 purification in IMAC-Sepharose. Lane 1: molecular marker (kDa); lane 2: clarified homogenate; lane 3: NiF1, flow-through; lane 4: NiF2, wash; lane 5: NiF3, elution.

Fig. 7. SDS-PAGE of rfPspA245 purification in Q-Sepharose. Lane 1: molecular marker (kDa); lane 2: Q loading fraction; lane 3: QF1, flow-through; lane 4: QF2, wash 1; lane 5: QF3, wash 2; lane 6: QF4, elution; lane 7: QF5, cleaning.

Table 2. Purification of rfPspA245 in IMAC-Sepharose followed by Q-Sepharose.

Sample	Total Protein (mg)	Relative Purity rfPspA245 (%) [#]	rfPspA245 (mg)	Recovery rfPspA245 (%)	Purification Factor
Clarified Homogenate	53872	44.0	23704	100.0	1.0
IMAC-Sepharose (NiF3)	4100	68.5	2808	11.8	1.5
Q- Sepharose (QF4)	2700	79.9	2157	9.1	1.8 (1.2)*

[#] Calculated by densitometry. * Value between parentheses is the purification factor of this step.

4. Conclusions

The PspA245 was produced in a high cell density cultivation of *E. coli*, the cells were disrupted with high efficiency and the best sequence for the purification of recombinant PspA was Q-Sepharose followed by IMAC-Sepharose.

The purification processes still need to be improved, especially in the recovery from IMAC chromatography and may be also in the recovery of Q-Sepharose, but it is noteworthy that PspA245 was obtained with purity of 96.6%.

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References

- [1] Gray BM, Turner ME, Dillon Jr HC. Epidemiologic studies of *Streptococcus pneumoniae* in infants: the effects of season and age on pneumococcal acquisition and carriage in the first 24 months of life. *Amer J Epidemiol* 1982;**116**:692-703.

- [2] Rosen C, Christensen P, Hovelius B. A longitudinal study of nasopharyngeal carriage of pneumococci related to pneumococcal vaccination in children attending day-care centers. *Acta Otolaryng* 1984;**98**:524-32.
- [3] Lee CJ, Banks SD, Lee JP. Virulence, Immunity, and vaccine related to *Streptococcus pneumoniae*. *Crit Rev Microbiol* 1991;**18**:89-114.
- [4] Brandileone MC, de Andrade AL, Di Fabio JL, Guerra ML, Austrian R. Appropriateness of a pneumococcal conjugate vaccine in Brazil: potential impact of age and clinical diagnosis, with emphasis on meningitis. *J Infect Dis* 2003;**187**:1206-12.
- [5] Spika JS, Facklam RR, Pikaytis BD, Oxtoby MJ, Party PSW. Antimicrobial resistance of *Streptococcus pneumoniae* in the United States, 1979-1987. *J Infect Dis* 1991;**163**:1273-8.
- [6] Wenger JD, Booy R, Heath PT, Moxon R. Epidemiological impact of conjugate vaccines on invasive disease caused by *Haemophilus influenzae* type b. In: Levine MM, Woodrow GC, Kaper JB, Cobon GS, editors. *New generation vaccines*. 2nd ed. New York: Marcel Dekker; 1997, p. 489-502
- [7] Schneerson R, Robbins JB, Parke JC. Quantitative and qualitative analyses of serum antibodies elicited in adults by *Haemophilus influenzae* type b and pneumococcus type 6A capsular polysaccharide-tetanus conjugates. *Infect Immun* 1986;**50**:519-28.
- [8] Eby R, Koster M, Hogerman D. Pneumococcal conjugates. In: Norrby E, Brown F, Chanock RM, Ginsberg HS, editors. *Vaccines '94: Modern approaches to new vaccines including prevention of AIDS*. New York: Cold Spring Harbor Laboratory Press; 1994, p. 119-24
- [9] Vella P, Marburg S, Staub J, Kniskern PJ, Milel W, Hagopian A, et al. Immunogenicity of conjugate vaccines consisting of pneumococcal polysaccharide type 6B, 14, 19F and 23F and a meningococcal outer membrane protein complex. *Infect Immun* 1992;**60**:4977-83.
- [10] Henrichsen J. Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* 1995;**33**:2759-62.
- [11] Siber GR. Pneumococcal disease: prospects for a new generation vaccine. *Science* 1994;**265**:1385-7.
- [12] Daniels CC, Briles TC, Mirza S, Anders P, Hakansson AP, Briles DE. Capsule does not block antibody binding to PspA, a surface virulence protein of *Streptococcus pneumoniae*. *Microb Pathog* 2006;**40**:228-33.
- [13] McDaniel LS, Ralph BA, McDaniel DO, Briles DE. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. *Microb Pathog* 1994;**17**:323-37.
- [14] Tai SS. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. *Crit Rev Microbiol* 2006; **32**:139-53.
- [15] Hollingshead SK, Becker RS, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. *Infect Immun* 2000; **68**:5889–900.
- [16] Shiloach J, Fass R. Growing *E. coli* to high cell density – a historical perspective on method development. *Biotechnol Adv* 2005; **23**:345-57.
- [17] Asenjo JA, Andrews BA. Protein purification using chromatography: selection of type, modeling and optimization of operating conditions. *J Mol Recognit* 2009, **22**:65-76.
- [18] Xindu G, Lili W. Liquid chromatography of recombinant proteins and protein drugs – review. *J Chromatog B* 2008;**866**:133-53.
- [19] Seeger A, Schneppe B, McCarthy JEG, Deckwer WD, Rinas U. Comparison of temperature-induced and isopropyl-beta-d-thiogalacto-pyranoside-induced synthesis of basic fibroblast growth-factor in high-cell-density cultures of recombinant *Escherichia coli*. *Enz Microb Technol* 1995;**17**:947-53.
- [20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;**72**:248-54.
- [21] Laemmli UK, Beguin F, Gujer-Kellenberger G. A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J Mol Biol* 1970; **47**:69-85.