Development of a non-invasive murine infection model for acute otitis media

K. Stol,¹ S. van Selm,¹ S. van den Berg,¹ H. J. Bootsma,¹ W. A. M. Blokx,² K. Graamans,³ E. L. G. M. Tonnaer³ and P. W. M. Hermans¹

Correspondence

P. W. M. Hermans

P.Hermans@cukz.umcn.nl

¹Department of Pediatrics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

²Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

³Department of Otorhinolaryngology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Otitis media (OM) is one of the most frequent diseases in childhood, and *Streptococcus pneumoniae* is among the main causative bacterial agents. Since current experimental models used to study the bacterial pathogenesis of OM have several limitations, such as the invasiveness of the experimental procedures, we developed a non-invasive murine OM model. In our model, adapted from a previously developed rat OM model, a pressure cabin is used in which a 40 kPa pressure increase is applied to translocate pneumococci from the nasopharyngeal cavity into both mouse middle ears. Wild-type pneumococci were found to persist in the middle ear cavity for 144 h after infection, with a maximum bacterial load at 96 h. Inflammation was confirmed at 96 and 144 h post-infection by IL-1 β and TNF- α cytokine analysis and histopathology. Subsequently, we investigated the contribution of two surface-associated pneumococcal proteins, the streptococcal lipoprotein rotamase A (SlrA) and the putative proteinase maturation protein A (PpmA), to experimental OM in our model. Pneumococci lacking the *slrA* gene, but not those lacking the *ppmA* gene, were significantly reduced in virulence in the OM model. Importantly, pneumococci lacking both genes were significantly more attenuated than the Δ *slrA* single mutant. This additive effect suggests that SlrA and PpmA exert complementary functions during experimental OM. In conclusion, we have developed a highly reproducible and non-invasive murine infection model for pneumococcal OM using a pressure cabin, which is very suitable to study pneumococcal pathogenesis and virulence *in vivo*.

Received 27 July 2009

Revised 15 September 2009

Accepted 16 September 2009

INTRODUCTION

In the first 3 years of life, otitis media (OM) affects 80 % of the paediatric population (Giebink, 2001). *Streptococcus pneumoniae*, together with non-typable *Haemophilus influenzae* and *Moraxella catarrhalis*, is considered to be one of the three major bacterial pathogens involved in OM (Lysenko *et al.*, 2005; Ryan *et al.*, 2006; Sabirov & Metzger, 2006, 2008). OM is the most common reason for children to visit a physician, to receive antibiotics or to undergo surgery, which, consequently, results in a high disease burden for both children and their parents. Treatment with antibiotics remains controversial and is complicated by the rising numbers of isolates resistant to antibiotics (Cripps *et al.*, 2005; Ogunniyi *et al.*, 2007). Despite the significant

burden of OM on public health, the genetic basis of bacterial OM remains largely unclear (Chen *et al.*, 2007).

Multiple animal models have been developed to study various aspects of the OM disease process, to increase our understanding of OM pathogenesis, and to design or improve preventive and treatment strategies (MacArthur & Trune, 2006). Different methods are used to provoke middle ear inflammation in various animal species: models using mice (Krekorian *et al.*, 1990; MacArthur & Trune, 2006; Ryan *et al.*, 2006; Sabirov & Metzger, 2008), rats (Fogle-Ansson *et al.*, 2006; Melhus & Ryan, 2003; Tonnaer *et al.*, 2003; van der Ven *et al.*, 1999), gerbils, guinea pigs, chinchillas (Ehrlich *et al.*, 2002; Fulghum & Marrow, 1996; Giebink *et al.*, 1980) and monkeys (Alper *et al.*, 2000) have previously been described in the literature. Among these, mice are increasingly becoming the model of choice in OM research. BALB/c mice are considered to be most susceptible to experimental acute otitis media (AOM)

Abbreviations: AOM, acute otitis media; CI, competitive index; IL, interleukin; OM, otitis media; PPlase, peptidyl-prolyl isomerase; TNF, tumour necrosis factor.

(MacArthur *et al.*, 2006; Melhus & Ryan, 2003; Ryan *et al.*, 2006; Sandgren *et al.*, 2005).

AOM can be evoked by the introduction of live or heat-killed bacteria, as well as by inflammatory substances (Ryan *et al.*, 2006; Sabirov & Metzger, 2008). In most animal models, AOM is induced by direct inoculation of live bacteria into the middle ear cavity, via either a transtympanic or a transbullar route. In the transtympanic model, application of the inoculum into the middle ear cavity is achieved by direct injection through the tympanic membrane. The transbullar approach consists of a ventral midline incision in the neck under sedation. The bulla is exposed after blunt dissection and the middle ear is infected with a bacterial suspension using a thin needle through the bony wall (Ryan *et al.*, 2006; Sabirov & Metzger, 2006). Another possibility is intranasal inoculation, where initial bacterial colonization occurs in the nasopharynx, which is followed by invasion of the middle ear cavity by the pathogen in about 50 % of cases. This model resembles the natural route of infection in humans, and colonization of the nasopharynx is highly reproducible. However, development of OM after intranasal challenge is random and, consequently, reproducibility is low (Ryan *et al.*, 2006; Sabirov & Metzger, 2008).

Reproducible induction of OM that maintains the physiological route of infection has been achieved in rats by Tonnaer *et al.* (2003). In their model, rats are inoculated intranasally, after which pneumococci are transferred to the middle ear cavity via the Eustachian tube by an increase of atmospheric pressure. In the current study, we significantly adapted this model for mice, allowing us to study pneumococcal OM in a reproducible and consistent fashion.

Furthermore, we used our model to examine the contribution of two surface-associated pneumococcal proteins to OM: the streptococcal lipoprotein rotamase A (SlrA) and the putative proteinase maturation protein A (PpmA). SlrA and PpmA share homology with two distinct

families of peptidyl-prolyl isomerases (PPIases). Both these surface-associated proteins are known to play a key role during pneumococcal infection (Hermans *et al.*, 2006; Overweg *et al.*, 2000). Previous research has shown a pivotal role of SlrA in adherence, colonization and immune evasion, whereas PpmA is involved in invasive disease and colonization of the nasopharyngeal cavity (Cron *et al.*, 2009; Hermans *et al.*, 2006; Overweg *et al.*, 2000).

METHODS

Pneumococcal strains and growth conditions. The pneumococcal strains used in this study are listed in Table 1, and were kindly provided by B. Henriques-Normark (Albiger *et al.*, 2005). *S. pneumoniae* was routinely grown in Todd-Hewitt broth supplemented with 0.5 % yeast extract or on Columbia blood agar (BA) plates at 37 °C and 5 % CO₂. Prior to mouse infection experiments, bacteria were passaged in mice to maintain virulence as described previously (Alexander *et al.*, 1994). Cultures of mouse-passaged *S. pneumoniae* strains were grown to an OD₆₂₀ of 0.2 (mid-exponential phase) and stored in aliquots at -80 °C in 15 % glycerol. For *in vivo* challenges, aliquots were spun down and bacteria were resuspended in sterile PBS containing 1 % methylcellulose, a viscous component, in order to minimize leakage of inoculum into the lungs (Tonnaer *et al.*, 2003). When indicated, antibiotics were used at the following concentrations: streptomycin, 100 µg ml⁻¹; trimethoprim, 25 µg ml⁻¹; and erythromycin, 4 µg ml⁻¹.

Construction of pneumococcal mutants. To obtain the streptomycin-resistant SME215::*rpsL*, the *rpsL* gene encoding a streptomycin-resistant mutant of the ribosomal protein S12 was amplified from D39::*rpsL* (Hermans *et al.*, 2006) with primer pair HBrsLF (GTACAGGGACGTGCTGACAA) and HBrsLR (CCCTTTCCTT-ATGCTTTTGG). The *rpsL*-PCR product was introduced into SME215 by CSP-1-induced transformation and selection for streptomycin resistance. To delete the *slrA* gene from SME215, the Δ *slrA* region was amplified from D39 Δ *slrA* (Hermans *et al.*, 2006) with primer pair HBslrAF (GTTCCCAAGCGAAATATGGA) and HBslrAR (TACACCAGGCGCTTTATCT). The Δ *slrA*-PCR product was introduced into SME215 by CSP-1-induced transformation and selection for erythromycin resistance. To obtain the SME215 Δ *ppmA* strain, a Δ *ppmA*-PCR region was amplified from D39 Δ *ppmA* (Adrian *et al.*, 2000) with primer pair HBppmAF1 (CTCTTGATGGCTG-

Table 1. Pneumococcal strains and mutants used in this study

Km^r, kanamycin resistance; Str^r, streptomycin resistance; Ery^r, erythromycin resistance; Trim^r, trimethoprim resistance; *slrA*, streptococcal lipoprotein rotamase A; *ppmA*, putative proteinase maturation protein A.

Strain	Serotype	Relevant characteristics	Reference
<i>S. pneumoniae</i>			
D39	2	–	NCTC 7466
TIGR4(lux)	4	Tn4001 <i>luxABCDE</i> operon, Km ^r	Albiger <i>et al.</i> (2007)
PJ1324(lux)	6B	Tn4001 <i>luxABCDE</i> operon, Km ^r	Sandgren <i>et al.</i> (2005)
SME215(lux)	19F	Tn4001 <i>luxABCDE</i> operon, Km ^r	Albiger <i>et al.</i> (2005), Sandgren <i>et al.</i> (2005)
SME215(lux) strep	19F	SME215(lux) derivative, <i>rpsL</i> ; Str ^r	This study
SME215(lux) Δ <i>slrA</i>	19F	SME215(lux) derivative, Δ <i>slrA</i> ; Ery ^r	This study
SME215(lux) Δ <i>ppmA</i>	19F	SME215(lux) derivative, Δ <i>ppmA</i> ; Trim ^r	This study
SME215(lux) Δ <i>slrAΔ<i>ppmA</i></i>	19F	SME215(lux) derivative, Δ <i>slrA</i> Δ <i>ppmA</i> ; Ery ^r Trim ^r	This study

AACATGC) and HBppmAR1 (GCAGCCTACAGCTAGCTTCC). The Δ ppmA-PCR product was introduced into SME215 by CSP-1 induced transformation, and trimethoprim-resistant transformants lacking the *ppmA* gene were selected (Adrian *et al.*, 2000; Hermans *et al.*, 2006). The Δ slrA Δ ppmA double mutants were constructed by transformation of the Δ ppmA-PCR product (amplified with primer pair HBppmAF1 and HBppmAR1) to each Δ slrA mutant and selecting for trimethoprim and erythromycin resistance. Deletion of *slrA* and *ppmA* in resulting transformants was confirmed by PCR and sequencing.

Mouse strain and disease monitoring. Experiments were conducted with 7-week-old, female, specific-pathogen-free BALB/c mice (Harlan). They conformed to Dutch animal protection legislation.

Pressure cabin. The pressure cabin consists of a perspex tube (30 cm, diameter 15 cm) with a fixed perspex wall. The perspex wall contains a manometer, an outlet valve, and a valve connected to a cylinder filled with compressed air. The removable front door is sealed with a rubber O-ring. Air pressure in the cabin can be increased stepwise (0–100 kPa) and monitored precisely (Tonnaer *et al.*, 2003).

Mouse OM model: single and co-infection. Mice were lightly anaesthetized with 2.5 % (v/v) isoflurane over oxygen (1.5 l min⁻¹), and subsequently, a 10 μ l suspension (containing 5×10^6 c.f.u. bacteria or PBS/1 % methylcellulose as control) was applied onto both nostrils in alternating fashion (single infection and co-infection). Mice were placed in supine position with an initial pressure rise set at 10 kPa. When the mice regained consciousness and the first swallowing movements occurred, pressure was raised to 40 kPa, at 5 kPa per 15 s, in order to transfer the inoculum to the middle ear cavity. Thereafter, the pressure was lowered stepwise until atmospheric pressure was reached again. Pressure increase was evaluated using otomicroscopy and the tympanic membrane was scored for tympanic membrane perforation and for increased vascularization or bleeding.

For single infection with *S. pneumoniae* D39, TIGR4, SME215 or PJ1324, groups of 5–9 mice were sacrificed at 96 h post-inoculation. For single infection with *S. pneumoniae* SME215, groups of five mice were sacrificed at 1.5, 48, 96, and 144 h post-inoculation. Blood was collected using orbital puncture. The bulla enclosing the right middle ear was dissected from the temporal bone and homogenized in 1 ml sterile PBS, as previously described (Kakiuchi *et al.*, 2006; Tonnaer *et al.*, 2003). Thereafter bacteria were recovered from the nasopharynx by flushing the nose with 1 ml sterile PBS per naris and collection at the contralateral side, a modified method from Wu *et al.* (1997). Both lungs were extracted and homogenized in 2 ml sterile PBS. Viable counts were determined by serial 10-fold dilutions plated on BA plates.

For co-infection, a 10 μ l inoculum containing a 1:1 ratio of streptomycin-resistant *S. pneumoniae* SME215 wild-type and either the Δ slrA, Δ ppmA or Δ slrA Δ ppmA mutant (5×10^6 total c.f.u.) was used to infect the mice intranasally as described above. Groups of five mice were sacrificed at 1.5, 12, 48 and 96 h post-infection, whereupon samples from ear, lungs, nasopharynx and blood were collected as described above. C.f.u. counts were determined by plating serial dilutions of the specimens on BA plates and Columbia agar plates supplemented with 5 % (v/v) defibrinated sheep blood (Biotrading) containing the appropriate antibiotics.

Co-infection reduces variation between individual mice, inoculation preparation and distribution, and sample collection. Competitive index (CI) scores were calculated for the co-infection experiments as previously described (Hava & Camilli, 2002). In short, the CI for each individual animal was calculated as the output ratio of mutant and wild-type divided by the input ratio of mutant to wild-type bacteria.

For experiments in which no mutant bacteria were recovered from a particular mouse, the number 20 (lower limit of detection) was used as the numerator. Mice with neither mutant nor wild-type bacteria in the middle ear were excluded from further analysis.

Histology. The skulls of all mice including both temporal bones, nose and maxilla were removed and processed for histological analysis. The specimens were formalin fixed in 4 % paraformaldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4), decalcified using 10 % EDTA (Serva), and embedded in paraffin. Subsequently, all skulls were cut into 5 mm slices in an oblique direction. After deparaffinization through a series of xylene and graded ethanol baths, sections were stained with haematoxylin/eosin. Sections were photographed with a microscope (Carl Zeiss Axioskop 2 plus) coupled to a computer (ProgRes Capturepro 2.1) at an original magnification of $25 \times$ and $200 \times$.

Interleukin (IL)-1 β and tumour necrosis factor (TNF)- α ELISA. Commercially available kits were used to measure the IL-1 β (Becton Dickinson) and TNF- α (U-cytech) concentrations in murine middle ear homogenate and nasopharyngeal lavage.

Statistical analysis. All data were analysed using SPSS 16.0 and GraphPad Prism version 4.0. For single infection a non-parametric test on log-transformed c.f.u. counts (mean, $P < 0.05$) was used to calculate statistical significance. For co-infection the one-sample *t*-test on log-transformed CI scores (with an arbitrary mean of 0 and $P < 0.01$) and comparative regression coefficients were used to calculate statistical significance.

Cytokine levels were calculated according to the manufacturer's instructions. A non-parametric test (mean, SEM and $P < 0.05$) was performed to calculate statistical significance between infected and PBS-infected mice at the corresponding time points.

RESULTS

Development of a non-invasive murine OM model

The pathogenesis of pneumococcal OM involves bacterial translocation from the nasopharynx to the middle ear cavity through the Eustachian tube. In order to translocate bacteria in our model, we used a pressure cabin. First, we identified the appropriate pressure in our model with Evans blue dye. A pressure increase of 40 kPa was sufficient to transfer Evans blue dye from the nasopharynx into the middle ear cavity of BALB/c mice, without causing damage to the tympanic membrane: otomicroscopy did not show rupture of the tympanic membrane, nor vascular dilatation of blood vessels present at the tympanic membrane (data not shown). Subsequently, the pressure cabin was used for bacterial infection with *S. pneumoniae* TIGR4 to test various doses and inoculum volumes. A dose of 5×10^6 c.f.u. in a 10 μ l volume resulted in reproducible infection, in line with the mouse colonization model used in our laboratory (Cron *et al.*, 2009; Hendriksen *et al.*, 2009). To examine strain-specific behaviour, mice were infected with the pneumococcal strains D39 (serotype 2), TIGR4 (serotype 4), PJ1324 (serotype 6B) and SME215 (serotype 19F). Ninety-six hours after infection mice were euthanized, whereupon ear and lung homogenates, nose lavage and blood were cultured. *S. pneumoniae* SME215

and PJ1324 induced a median bacterial load in the middle ear of 1×10^4 c.f.u. and 5×10^2 c.f.u. respectively, at 96 h post-inoculation (Fig. 1). OM induced by *S. pneumoniae* TIGR4 was complicated by pneumonia and sepsis (as confirmed by clinical scores and bacterial culture) and consequently excluded from further analysis. Counts of *S. pneumoniae* D39 in the middle ear and nasopharynx were significantly lower compared to *S. pneumoniae* SME215 (P 0.003) and PJ1324 (P 0.039). Therefore, *S. pneumoniae* PJ1324 and SME215 were considered most suitable for our model. No bacteria or signs of inflammation were detected in middle ears of mice inoculated with PBS, indicating that transfer of local nasopharyngeal flora does not occur in our model.

Since the bacterial load of *S. pneumoniae* SME215 in the middle ear was significantly higher than that of *S. pneumoniae* PJ1324 (P 0.015), SME215 was used to examine the bacterial kinetics in further detail at 1.5, 48, 96 and 144 h after infection (Fig. 2). Approximately 1×10^5 c.f.u. were detected in the middle ear cavity at 1.5 h post-inoculation; this number declined 50-fold at 48 h post-inoculation. Wild-type pneumococci persisted in the middle ear cavity up to 96 h post-infection (median 3×10^4 c.f.u.). The bacterial load in the nasopharynx reached 1×10^6 c.f.u. up to 96 h post-inoculation and 4×10^5 c.f.u. at 144 h. The bacterial load initially detected in the lungs was high, 1×10^6 c.f.u., but declined rapidly over time, with 5×10^2 c.f.u. at 48 h and 6×10^1 c.f.u. at 96 h post-inoculation (Fig. 2). No bacteria were recovered from blood (data not shown). Weight and temperature of the mice remained within the normal range and, importantly, mice did not show any signs of invasive

disease; in particular no signs of pneumonia and sepsis were observed.

Immunology and histopathology

Both IL-1 β and TNF- α levels in the middle ear were elevated throughout the course of infection. Importantly, levels in PBS-inoculated mice were below the detection limit at 1.5, 48 and 96 h post-inoculation and marginal at 144 h post-inoculation (TNF- α , 14.5 pg ml $^{-1}$; IL-1 β , 24.4 pg ml $^{-1}$). The levels of TNF- α and IL-1 β in the middle ear at 48 h and 96 h post-inoculation were significantly different from those in PBS-inoculated mice collected at the corresponding time points (Fig. 3A, B). The TNF- α cytokine levels detected in the nasopharynx were 10-fold lower than the levels in the middle ear, and significantly different at 48, 96 and 144 h post-inoculation when compared to PBS-inoculated mice (Fig. 3C). There was no significant difference between infected and PBS-infected mice in the IL-1 β levels in the nasopharynx (Fig. 3D).

Histopathological examination showed an increased inflammation over time (Fig. 4). Neutrophils, lymphocytes and macrophages migrated into the middle ear cavity at 96 and 144 h post-inoculation (Fig. 4C, D). The mucous membrane, which is normally lined by a single layer of epithelium, by then was covered with a more irregular and multiple-layered epithelium (Fig. 4C, b and c; Fig. 4D, b and c). The loose connective tissue had expanded significantly, due to the presence of transient wandering immune cells that had migrated from local blood vessels to the site of infection (Fig. 4C, b and c; Fig. 4D, b and c).

Fig. 1. Bacterial load in the middle ear (A), nasopharyngeal lavage (B), lungs (C) and blood (D) 96 h after inoculation with *S. pneumoniae* D39 (serotype 2), TIGR4 (serotype 4), PJ1324 (serotype 6B) and SME215 (serotype 19F). Each dot represents a single mouse. Mean values are represented by horizontal bars. The lower limit of detection, 20 c.f.u., is indicated by a dashed line.

Fig. 2. Bacterial load in the middle ear (A), nasopharynx (B) and lungs (C) at various times after inoculation with *S. pneumoniae* SME215. Each dot represents one mouse. Mean values are indicated by horizontal bars. The lower limit of detection, 20 c.f.u., is indicated by a dashed line. No bacterial counts were detected in blood.

Usefulness of the murine OM model to study pneumococcal virulence

To assess the role of the pneumococcal proteins SlrA and PpmA in OM, we inactivated the *slrA* and *ppmA* genes in strain SME215 by insertion deletion mutagenesis, and tested the wild-type and $\Delta slrA$, $\Delta ppmA$ or $\Delta slrA\Delta ppmA$ mutants in a co-infection setup. Importantly, no differences in *in vitro* growth between the wild-type strains and the mutants were detected, indicating that differences in virulence did not originate from altered growth rates.

In the middle ear, the bacterial load of the $\Delta slrA$ mutant was significantly lower relative to the SME215 parent strain

at 48 and 96 h post-infection (mean CI 0.14 and 0.07 respectively, a 10-fold reduction) (Fig. 5A). A significant difference between the wild-type and the $\Delta ppmA$ mutant was only found at 1.5 h after infection, not at later time points (mean CI 0.47, a fivefold reduction) (Fig. 5C). Deletion of both *slrA* and *ppmA* resulted in a significant decrease in middle ear bacterial load compared to wild-type at all time points: the CI score declined from 0.26 at 1.5 h post-inoculation to 0.003 at 96 h post-inoculation, representing a 3- to 300-fold reduction over time. In other words, the wild-type outcompeted the double knockout strain in a progressive and statistically significant fashion (Fig. 5E). Interestingly, we observed an additive effect upon

Fig. 3. IL-1 β and TNF- α cytokine levels in the middle ear and nasopharynx. Each bar represents the mean value of five mice, error bars indicate SEM. (A) TNF- α in the middle ear; (B) IL-1 β in the middle ear; (C) TNF- α in the nasopharyngeal lavage; (D) IL-1 β in the nasopharyngeal lavage. Statistical significance was calculated between infected and mock-infected mice at the corresponding time point of collection. Significance ($P < 0.05$) is depicted by *.

Fig. 4. Histopathological changes in the middle ear. Sections were photographed at an original magnification of 25 $\times$ and 200 $\times$ respectively. (A) Normal, non-infected middle ear; (B) 48 h after intranasal inoculation with *S. pneumoniae* SME215; (C) 96 h after intranasal inoculation with *S. pneumoniae* SME215; (D) 144 h after intranasal inoculation with *S. pneumoniae* SME215. The mucous membrane, which is normally lined by a single layer of epithelium (panel A, b and c), by then is covered with a more irregular and multiple layered epithelium (panel C, b and c; panel D, b and c). The loose connective tissue (panel A, b and c) has expanded significantly, due to the presence of transient wandering immune cells that have migrated from local blood vessels to the site of infection (panel C, b and c; panel D, b and c). Abbreviations: OE, outer ear; TM, tympanic membrane; MEC, middle ear cavity; MM, mucous membrane; ET, Eustachian tube.

deletion of both genes, as the $\Delta slrA\Delta ppmA$ mutant was significantly more attenuated in OM virulence than the $\Delta slrA$ or $\Delta ppmA$ single mutant (Fig. 5A, C, E).

Colonization of the nasopharynx was highly reproducible, yielding approximately 5×10^5 c.f.u. at all time points. Throughout the course of the experiment, the $\Delta slrA$, $\Delta ppmA$ and $\Delta slrA\Delta ppmA$ mutants were less able to colonize the nasopharynx in comparison to the parent strain (Fig. 5B, D, F). Deletion of *slrA* resulted in a significant difference in colonization of the nasopharynx from wild-type at all time points (mean CI varying from 0.06 to 0.14, a 7- to 17-fold reduction) (Fig. 5B). A significant difference between the wild-type and the $\Delta ppmA$ mutant was seen at 12 h (mean CI 0.70), 48 h

(mean CI 0.26) and 96 h (mean CI 0.15) h post-inoculation (Fig. 5D). In line with the results for the $\Delta slrA\Delta ppmA$ mutant in the middle ear, outcompetition of the double mutant in the nasopharynx was progressive and statistically significant over time (CI 0.10–0.01, a 10- to 100-fold reduction) (Fig. 5F). Again, the $\Delta slrA\Delta ppmA$ mutant was significantly more attenuated in virulence than the $\Delta slrA$ or $\Delta ppmA$ single mutant (Fig. 5B, D, F).

DISCUSSION

In the present study we developed a novel non-invasive murine OM model with the use of a pressure cabin. The mouse affords many advantages for *in vivo* research,

Fig. 5. Competitive index (CI) in the middle ear (A, C, E) and nasopharynx (B, D, F) after co-infection, using the murine OM model. Female BALB/c mice were infected intranasally with a 1:1 mix of *S. pneumoniae* SME215 wild-type and *S. pneumoniae* SME215ΔslrA (A, B), *S. pneumoniae* SME215 wild-type and *S. pneumoniae* SME215ΔppmA (C, D) and *S. pneumoniae* SME215 wild-type and *S. pneumoniae* SME215ΔslrAΔppmA (E, F). Each square represents one mouse. The CI score of 1 (i.e. no difference between wild-type and mutant) is indicated by a dashed line. Statistical significance of $P < 0.01$ is depicted by **.

including ease of genetic manipulation, availability of inbred and transgenic strains and an extensively studied immune system. Moreover, experimental reagents for cellular and molecular studies are widely available (Krekorian *et al.*, 1990; MacArthur & Trune, 2006; Ryan *et al.*, 2006; Sabirov & Metzger, 2008). The model, which was adapted from a rat OM model described by Tonnaer *et al.* (2003), was used to investigate OM caused by *S. pneumoniae* and the contribution of the pneumococcal proteins SlrA and PpmA to the pathogenesis of OM.

For various reasons, the pressure cabin model is an improvement over the experimental AOM models currently used. First, pressure elevation facilitates the ascending infection of pneumococci from the nasopharynx to the middle ear cavity via the Eustachian tube, resembling the natural route of infection in humans. In comparison to AOM models, which rely on spontaneous OM development after nasopharyngeal colonization (Sabirov & Metzger, 2008), the percentage of successful middle ear infections is 100% in our model. This is of major importance for future vaccination and challenge studies, since a 100% successful infection rate is required to monitor the efficiency of a vaccine, i.e. reduction of bacterial load. Second, dermal pathogens like

Staphylococcus epidermidis will not influence experimental results, whereas with the widely used transtympanic or transbullar approaches, a connection between the middle ear and its surroundings is established. Third, an inflammatory response due to local manipulation is avoided. Among others, MacArthur *et al.* (2006) described signs of inflammation due to direct instillation of PBS in the middle ear that were equivalent to those induced by lower doses of heat-killed bacteria. These inflammatory reactions were ascribed to the required tympanic membrane incision (Krekorian *et al.*, 1990; MacArthur *et al.*, 2006).

Of the pneumococcal strains tested, BALB/c mice were most susceptible to TIGR4 (serotype 4), followed by SME215 (serotype 19F), PJ1324 (serotype 6B) and D39 (serotype 2). These observations indicate pneumococcal strain-specific behaviour *in vivo* as described previously (Briles *et al.*, 1992; Forbes *et al.*, 2008). *S. pneumoniae* SME215 and PJ1324 persisted in the middle ear cavity for at least 96 h. These two strains were therefore considered suitable for experimental OM studies *in vivo*, and subsequent experiments were performed using *S. pneumoniae* SME215 only. On the day of inoculation with *S. pneumoniae* SME215, bacteria were detected in the lungs,

but they were cleared rapidly over time. We speculate that leakage to the lungs can be partially explained by the use of anaesthetics and pressure increase at the time of infection. Importantly, disease scores were low during this study, which implies absence of pneumonia or sepsis, and – as a result – moribund state or early loss did not occur. This is in contrast to current chinchilla OM models, which often record a moribund disease state (Forbes *et al.*, 2008). Since signs and symptoms of human OM mostly show a mild course of infection (Cripps *et al.*, 2005), we consider our model to be a clinical representation of OM in humans. Moreover, infection with heat-inactivated bacteria to avoid systemic complications, as described in OM mouse models using direct tympanic membrane inoculation, was not necessary (MacArthur *et al.*, 2006). Polymicrobial interactions between, for instance, *S. pneumoniae*, (non-typable) *H. influenzae* and *M. catarrhalis*, or combined bacterial and viral infections are of interest for future studies (Bakaletz, 2004; Cripps *et al.*, 2005; Lysenko *et al.*, 2005; MacArthur *et al.*, 2006).

The primary cytokines TNF- α and IL-1 β were selected to monitor the initiation of the acute inflammatory response. TNF- α is produced mainly by macrophages or mast cells and is, among others, responsible for neutrophil migration (Maeda *et al.*, 2004). Along with various other functions, IL-1 β contributes to permanent pathological changes in the middle ear, mucosal damage, bone erosion, fibrosis and hearing loss. Higher concentrations of IL-1 β were observed in purulent, acute, culture-positive effusions (Maeda *et al.*, 2004; Skotnicka & Hassmann, 2008; Tong *et al.*, 2008). Our results indicate a peak TNF- α and IL-1 β response in the middle ear cavity at 96 h post-inoculation, whereas the maximum histopathological changes were shown at 144 h post-inoculation. These data confirm ongoing inflammation after onset of the inflammatory process by the primary cytokines: at 144 h, levels of both TNF- α and IL-1 β were comparable to PBS-treated mice, most likely since the number of white blood cells was sufficient to eradicate *S. pneumoniae* from the middle ear cavity. Using a rat OM model Cripps & Kyd (2007) reported a maximum TNF- α level of 1.3×10^3 pg ml $^{-1}$ at 24 h post-infection, while IL-1 β was not detectable. They also reported cytokine levels in a mouse pneumonia model of pneumococcal infection: a maximum TNF- α level around 3.0×10^2 pg ml $^{-1}$ at 8 h post-infection and maximum IL-1 β level near 6.5×10^2 pg ml $^{-1}$ at 24 h post-infection (Cripps & Kyd, 2007). However, as immune responses in *in vivo* experimental models are animal specific, bacterial-strain specific, and infection-site specific, it is difficult to directly compare the results. Unlike the strong inflammation in the middle ear (Tuomanen, 2000), there is a relatively mild host response to colonizing pneumococci in the nasopharynx (Chen *et al.*, 2007), underscored by the marginal levels of IL-1 β and TNF- α detected in the nasopharyngeal lavage in our study.

Bacterial surface-exposed proteins often play an important role in the interaction between pathogens and their host. Identification of novel surface-exposed proteins that play

an important role in virulence should improve our understanding of OM pathogenesis and will facilitate new preventive strategies such as vaccine development. Virulence studies described in the literature have shown that most pneumococcal mutants display attenuated phenotypes in colonization, pneumonia, or sepsis mouse models, thus indicating niche-specific involvement in virulence of the respective genes (Chen *et al.*, 2007; Cripps *et al.*, 2005; Hava & Camilli, 2002; Hendriksen *et al.*, 2007, 2008; Hermans *et al.*, 2006; Ogunniyi *et al.*, 2007; Tong *et al.*, 2004; Tuomanen, 2000).

To our knowledge we report here the first OM virulence study performed in mice. Only very few studies have investigated the role of virulence factors in OM models (Chen *et al.*, 2007; Tong *et al.*, 2004, 2008). Chen *et al.* (2007) very nicely demonstrated the first extensive search for genetic requirements for pneumococcal OM by using signature-tagged mutagenesis in a chinchilla OM model and a murine colonization model. Since direct extrapolation from other models is problematic, given the animal-model specificity (Chen *et al.*, 2007), we examined the contribution of two factors to OM and nasopharyngeal colonization directly. We showed that both single gene deletion of *slrA* and combined deletion of the *slrA* and *ppmA* genes significantly reduced the bacterial load during experimental AOM in both the nasopharynx and the middle ear compared to the wild-type strain. Pneumococci lacking the *slrA* gene, but not those lacking the *ppmA* gene, were significantly reduced in virulence in the OM model. Interestingly, the virulence of the pneumococci lacking both genes was significantly decreased compared to the Δ *slrA* or Δ *ppmA* single mutant. This observation suggests complementary functions of SlrA and PpmA in both experimental OM and nasopharyngeal colonization, resulting in an additive decrease in virulence when both genes are absent. Previous studies have shown a role for both SlrA and PpmA in the early stage of infection, i.e. colonization (Cron *et al.*, 2009; Hermans *et al.*, 2006). Our results, even in a different mouse background and with a different pneumococcal strain, confirmed these findings. SlrA is a functional PPLase involved in pneumococcal colonization, most likely by modulating the biological function of important virulence proteins, as described by Hermans *et al.* (2006). Although PpmA is a conserved surface protein with potential to elicit protective immune responses, the exact role of this protein is still unknown (Overweg *et al.*, 2000).

In summary, we have developed an OM mouse model in which AOM can be established by various pneumococcal strains. This highly reproducible method is non-invasive, and infection is established in both ears. Consequently, multiple simultaneous applications are feasible, such as bacterial culture and histopathology. The model will be highly valuable for studying OM induced by *S. pneumoniae*, and OM-related pneumococcal virulence. It is also likely to be suitable for investigating the protective capacity of putative vaccine antigens against pneumococcal-induced

OM, since the route of infection does not bypass local immunity and the middle ear infection rate is 100 %. Whether this model is also appropriate for other pathogens such as *H. influenzae* and *M. catarrhalis* is currently under investigation.

ACKNOWLEDGEMENTS

This project was funded by the EC Sixth Framework Programme. We thank B. Henriques-Normark for providing the *S. pneumoniae* strains TIGR4, PJ1324 and SME215, and E. Simonetti and F. Hendriks for technical assistance. A. Maat is acknowledged for histological assistance.

REFERENCES

- Adrian, P. V., Thomson, C. J., Klugman, K. P. & Amyes, S. G. (2000). New gene cassettes for trimethoprim resistance, *dfp13*, and streptomycin-spectinomycin resistance, *aadA4*, inserted on a class 1 integron. *Antimicrob Agents Chemother* **44**, 355–361.
- Albiger, B., Sandgren, A., Katsuragi, H., Meyer-Hoffert, U., Beiter, K., Wartha, F., Hornef, M., Normark, S. & Normark, B. H. (2005). Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. *Cell Microbiol* **7**, 1603–1615.
- Albiger, B., Dahlberg, S., Sandgren, A., Wartha, F., Beiter, K., Katsuragi, H., Akira, S., Normark, S. & Henriques-Normark, B. (2007). Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cell Microbiol* **9**, 633–644.
- Alexander, J. E., Lock, R. A., Peeters, C. C., Poolman, J. T., Andrew, P. W., Mitchell, T. J., Hansman, D. & Paton, J. C. (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.
- Alper, C. M., Swarts, J. D. & Doyle, W. J. (2000). Prevention of otitis media with effusion by repeated air inflation in a monkey model. *Arch Otolaryngol Head Neck Surg* **126**, 609–614.
- Bakaletz, L. O. (2004). Developing animal models for polymicrobial diseases. *Nat Rev Microbiol* **2**, 552–568.
- Briles, D. E., Crain, M. J., Gray, B. M., Forman, C. & Yother, J. (1992). Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. *Infect Immun* **60**, 111–116.
- Chen, H., Ma, Y., Yang, J., O'Brien, C. J., Lee, S. L., Mazurkiewicz, J. E., Haataja, S., Yan, J. H., Gao, G. F. & Zhang, J. R. (2007). Genetic requirement for pneumococcal ear infection. *PLoS One* **3**, e2950.
- Cripps, A. W. & Kyd, J. M. (2007). Comparison of mucosal and parenteral immunisation in two animal models of pneumococcal infection: otitis media and acute pneumonia. *Vaccine* **25**, 2471–2477.
- Cripps, A. W., Otczyk, D. C. & Kyd, J. M. (2005). Bacterial otitis media: a vaccine preventable disease? *Vaccine* **23**, 2304–2310.
- Cron, L. E., Bootsma, H. J., Noske, N., Burghout, P., Hammerschmidt, S. & Hermans, P. W. (2009). Surface-associated lipoprotein PpmA of *Streptococcus pneumoniae* is involved in colonization in a strain-specific manner. *Microbiology* **155**, 2401–2410.
- Ehrlich, G. D., Veeh, R., Wang, X., Costerton, J. W., Hayes, J. D., Hu, F. Z., Daigle, B. J., Ehrlich, M. D. & Post, J. C. (2002). Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* **287**, 1710–1715.
- Fogle-Ansson, M., White, P., Hermansson, A. & Melhus, A. (2006). Otomicroscopic findings and systemic interleukin-6 levels in relation to etiologic agent during experimental acute otitis media. *APMIS* **114**, 285–291.
- Forbes, M. L., Horsey, E., Hiller, N. L., Buchinsky, F. J., Hayes, J. D., Compliment, J. M., Hillman, T., Ezzo, S., Shen, K. & other authors (2008). Strain-specific virulence phenotypes of *Streptococcus pneumoniae* assessed using the *Chinchilla laniger* model of otitis media. *PLoS ONE* **3**, e1969.
- Fulghum, R. S. & Marrow, H. G. (1996). Experimental otitis media with *Moraxella (Branhamella) catarrhalis*. *Ann Otol Rhinol Laryngol* **105**, 234–241.
- Giebink, G. S. (2001). The prevention of pneumococcal disease in children. *N Engl J Med* **345**, 1177–1183.
- Giebink, G. S., Berzins, I. K., Marker, S. C. & Schiffman, G. (1980). Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and influenza A virus in chinchillas. *Infect Immun* **30**, 445–450.
- Hava, D. L. & Camilli, A. (2002). Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol Microbiol* **45**, 1389–1406.
- Hendriksen, W. T., Silva, N., Bootsma, H. J., Blue, C. E., Paterson, G. K., Kerr, A. R., de Jong, A., Kuipers, O. P., Hermans, P. W. & Mitchell, T. J. (2007). Regulation of gene expression in *Streptococcus pneumoniae* by response regulator 09 is strain dependent. *J Bacteriol* **189**, 1382–1389.
- Hendriksen, W. T., Kloosterman, T. G., Bootsma, H. J., Estevao, S., de Groot, R., Kuipers, O. P. & Hermans, P. W. (2008). Site-specific contributions of glutamine-dependent regulator GlnR and GlnR-regulated genes to virulence of *Streptococcus pneumoniae*. *Infect Immun* **76**, 1230–1238.
- Hendriksen, W. T., Bootsma, H. J., van Diepen, A., Estevao, S., Kuipers, O. P., de Groot, R. & Hermans, P. W. (2009). Strain-specific impact of PsaR of *Streptococcus pneumoniae* on global gene expression and virulence. *Microbiology* **155**, 1569–1579.
- Hermans, P. W., Adrian, P. V., Albert, C., Estevao, S., Hoogenboezem, T., Luijendijk, I. H., Kamphausen, T. & Hammerschmidt, S. (2006). The streptococcal lipoprotein rotamase A (SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization. *J Biol Chem* **281**, 968–976.
- Kakiuchi, M., Tsujigiwa, H., Orita, Y., Nagatsuka, H., Yoshinobu, J., Kariya, S., Haginomori, S., Orita, S., Fukushima, K. & other authors (2006). Cyclooxygenase 2 expression in otitis media with effusion. *Am J Otolaryngol* **27**, 81–85.
- Krekorian, T. D., Keithley, E. M., Takahashi, M., Fierer, J. & Harris, J. P. (1990). Endotoxin-induced otitis media with effusion in the mouse. Immunohistochemical analysis. *Acta Otolaryngol* **109**, 288–299.
- Lysenko, E. S., Ratner, A. J., Nelson, A. L. & Weiser, J. N. (2005). The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. *PLoS Pathog* **1**, e1.
- MacArthur, C. J. & Trune, D. R. (2006). Mouse models of otitis media. *Curr Opin Otolaryngol Head Neck Surg* **14**, 341–346.
- MacArthur, C. J., Hefeneider, S. H., Kempton, J. B., Parrish, S. K., McCoy, S. L. & Trune, D. R. (2006). Evaluation of the mouse model for acute otitis media. *Hear Res* **219**, 12–23.
- Maeda, K., Hirano, T., Ichimiya, I., Kurono, Y., Suzuki, M. & Mogi, G. (2004). Cytokine expression in experimental chronic otitis media with effusion in mice. *Laryngoscope* **114**, 1967–1972.
- Melhus, A. & Ryan, A. F. (2003). A mouse model for acute otitis media. *APMIS* **111**, 989–994.

- Ogunniyi, A. D., Grabowicz, M., Briles, D. E., Cook, J. & Paton, J. C. (2007). Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect Immun* **75**, 350–357.
- Overweg, K., Kerr, A., Sluijter, M., Jackson, M. H., Mitchell, T. J., de Jong, A. P., de Groot, R. & Hermans, P. W. (2000). The putative proteinase maturation protein A of *Streptococcus pneumoniae* is a conserved surface protein with potential to elicit protective immune responses. *Infect Immun* **68**, 4180–4188.
- Ryan, A. F., Ebmeyer, J., Furukawa, M., Pak, K., Melhus, A., Wasserman, S. I. & Chung, W. H. (2006). Mouse models of induced otitis media. *Brain Res* **1091**, 3–8.
- Sabirov, A. & Metzger, D. W. (2006). Intranasal vaccination of neonatal mice with polysaccharide conjugate vaccine for protection against pneumococcal otitis media. *Vaccine* **24**, 5584–5592.
- Sabirov, A. & Metzger, D. W. (2008). Mouse models for the study of mucosal vaccination against otitis media. *Vaccine* **26**, 1501–1524.
- Sandgren, A., Albiger, B., Orihuela, C. J., Tuomanen, E., Normark, S. & Henriques-Normark, B. (2005). Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans. *J Infect Dis* **192**, 791–800.
- Skotnicka, B. & Hassmann, E. (2008). Proinflammatory and immunoregulatory cytokines in the middle ear effusions. *Int J Pediatr Otorhinolaryngol* **72**, 13–17.
- Tong, H. H., Long, J. P., Li, D. & DeMaria, T. F. (2004). Alteration of gene expression in human middle ear epithelial cells induced by influenza A virus and its implication for the pathogenesis of otitis media. *Microb Pathog* **37**, 193–204.
- Tong, H. H., Chen, Y., Liu, X. & DeMaria, T. F. (2008). Differential expression of cytokine genes and iNOS induced by nonviable nontypeable *Haemophilus influenzae* or its LOS mutants during acute otitis media in the rat. *Int J Pediatr Otorhinolaryngol* **72**, 1183–1191.
- Tonnaer, E. L., Sanders, E. A. & Curfs, J. H. (2003). Bacterial otitis media: a new non-invasive rat model. *Vaccine* **21**, 4539–4544.
- Tuomanen, E. I. (2000). Pathogenesis of pneumococcal inflammation: otitis media. *Vaccine* **19** (Suppl 1), S38–S40.
- van der Ven, L. T., van den Dobbelsteen, G. P., Nagarajah, B., van Dijken, H., Dortant, P. M., Vos, J. G. & Roholl, P. J. (1999). A new rat model of otitis media caused by *Streptococcus pneumoniae*: conditions and application in immunization protocols. *Infect Immun* **67**, 6098–6103.
- Wu, H. Y., Virolainen, A., Mathews, B., King, J., Russell, M. W. & Briles, D. E. (1997). Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb Pathog* **23**, 127–137.

Edited by: M. Kilian