

Intranasal vaccination with γ -irradiated *Streptococcus pneumoniae* whole-cell vaccine provides serotype-independent protection mediated by B-cells and innate IL-17 responses

Rachelle Babb*, Austen Chen*, Timothy R. Hirst*†, Ervin E. Kara*, Shaun R. McColl*, Abiodun D. Ogunniyi*^{1,2}, James C. Paton*² and Mohammed Alsharifi*†²

*Research Centre for Infectious Diseases, Department of Molecular and Cellular Biology, School of Biological Sciences, University of Adelaide, Adelaide, SA 5005, Australia

†Gamma Vaccines Pty Ltd, Mountbatten Park, Yarralumla, ACT 2600, Australia

Abstract

Generating a pneumococcal vaccine that is serotype independent and cost effective remains a global challenge. γ -Irradiation has been used widely to sterilize biological products. It can also be utilized as an inactivation technique to generate whole-cell bacterial and viral vaccines with minimal impact on pathogen structure and antigenic determinants. In the present study, we utilized γ -irradiation to inactivate an un-encapsulated *Streptococcus pneumoniae* strain Rx1 with an unmarked deletion of the autolysin gene *lytA* and with the pneumolysin gene *ply* replaced with an allele encoding a non-toxic pneumolysoid (PdT) (designated γ -PN vaccine). Intranasal vaccination of C57BL/6 mice with γ -PN was shown to elicit serotype-independent protection in lethal challenge models of pneumococcal pneumonia and sepsis. Vaccine efficacy was shown to be reliant on B-cells and interleukin (IL)-17A responses. Interestingly, immunization promoted IL-17 production by innate cells not T helper 17 (Th17) cells. These data are the first to report the development of a non-adjuvanted intranasal γ -irradiated pneumococcal vaccine that generates effective serotype-independent protection, which is mediated by both humoral and innate IL-17 responses.

Key words: protective immunity, serotype independent, *Streptococcus pneumoniae*, vaccine.

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is one of the most challenging upper respiratory pathogens of humans, known to be the leading cause of otitis media, pneumonia, bacteraemia and meningitis [1]. It is also a common component of the nasopharyngeal microflora, and it is estimated that *S. pneumoniae* is carried asymptotically by approximately 60% of the population, with carriage rates higher among children under 6 years old [2]. Generally, factors such as an immuno-compromised host and co-infection with other pathogens trigger progression of asymptomatic infection into invasive disease. There are at least 1.2 million childhood deaths associated with *S. pneumoniae* per year, exem-

plifying the severity of pneumococcal disease on a global scale [1,3].

The capsular polysaccharides (CPS) are considered the major virulence factor of *S. pneumoniae* and have been the primary target for vaccination. Given the extensive number of *S. pneumoniae* capsular serotypes (93 identified to date), the ability to manufacture a universal pneumococcal vaccine remains a substantial challenge. One of the first pneumococcal vaccines licensed was the polyvalent polysaccharide vaccine, which covered initially 14, and later 23 of the most common circulating serotypes [4]. This vaccine is immunogenic in adults and children older than 5 years, but is poorly immunogenic in children less than 5 years old [5]. Lack of protection in younger children was related to

Abbreviations: CFU, colony forming units; CPS, capsular polysaccharide; IFN- γ , interferon- γ ; IL, interleukin; Lm, *Listeria monocytogenes*; LytA, autolysin; PdT, pneumolysoid; PspA, pneumococcal surface protein A; Th17, T helper 17 cell; TRM, tissue resident memory; WT, wild type; γ -PN, γ -irradiated *Streptococcus pneumoniae*; $\gamma\delta$ T17, IL-17⁺ $\gamma\delta$ T-cells.

¹ Present address: School of Animal and Veterinary Sciences, The University of Adelaide, SA 5005, Australia.

² These authors contributed equally to this work.

Correspondence: Mohammed Alsharifi (email mohammed.alsharifi@adelaide.edu.au) or James C. Paton (james.paton@adelaide.edu.au).

the type of immune response induced by CPS based vaccines, as CPS are T-cell-independent antigens and do not induce robust long-term humoral memory. These limitations led to development of pneumococcal conjugate vaccines, whereby the CPS is conjugated to a protein carrier to promote induction of T-cell-dependent immunity. These preparations elicit strong immunity in infants and young children [6]. The most recent conjugate vaccine (Pneumovax 13) covers serotypes and has been in routine use since 2010. However, current formulations are limited in their serotype coverage, which has increased prevalence of disease caused by non-vaccine serotypes, a phenomenon known as ‘serotype replacement’. In addition, high cost and limited technology restricts the number of serotypes that can be included in existing vaccine combinations [1,7].

In light of existing scientific challenges associated with pneumococcal disease, there is a clear need to develop serotype-independent pneumococcal vaccines. Whole un-encapsulated bacterial cells, as opposed to selected antigens, have recently been proposed as serotype-independent vaccine candidates [8]. Serotype-independent protection has been related to the induction of protective T helper 17 (Th17) cell-derived interleukin (IL)-17 responses. In fact, the importance of IL-17 responses in vaccine efficacy is becoming more apparent and has also been demonstrated for other pneumococcal vaccines [9–12]. However, common approaches for inactivation of whole cell-based vaccines include heat or ethanol treatment. Generally, both of these methods have been reported to affect membrane integrity of bacterial cells and subsequently impact vaccine immunogenicity [13,14]. Therefore, alternative inactivation methods are required to maintain pneumococcal antigens in their native form and to enhance vaccine immunogenicity.

We have been investigating the possibility of using γ -irradiation to inactivate pathogens for vaccine purposes [15]. γ -Irradiation involves the use of high frequency electromagnetic radiation referred to as ‘ γ -rays’, and has been used world-wide as a form of sterilization for food [16] and pharmaceuticals [17], and to inactivate highly infectious agents such as Ebola, Marburg and Lassa viruses [18]. γ -Irradiation has also been adopted as an inactivation method for both viral and bacterial vaccines due to reduced structural damage to antigenic epitopes and the ability to induce protective immune responses [14,19,20]. In the present study, we utilized γ -irradiation to inactivate an un-encapsulated whole-cell *S. pneumoniae* strain Rx1. We have also deleted the autolysin gene (*lytA*) and replaced the pneumolysin gene (*ply*) with an allele encoding a non-toxic pneumolysoid (PdT). LytA is responsible for cell lysis during growth of pneumococcal cells, whereas Ply is a pore forming toxin that plays many roles in *S. pneumoniae* pathogenesis including a major role in immune evasion (see review [1]). Highly concentrated stocks of the engineered pneumococcus were inactivated using γ -irradiation and used as a vaccine [designated γ -irradiated *S. pneumoniae* (γ -PN)]. In the present study, we demonstrate that intranasal vaccination with non-adjuvanted γ -PN vaccine induces serotype-independent protection against lethal intranasal challenge models of focal pneumonia and sepsis. Using gene-deficient mice and neutralizing antibody approaches, we show that serotype-independent protection elicited by γ -PN is dependent on B-cell

and IL-17 responses. Notably, γ -PN vaccination does not induce circulating or tissue-resident memory Th17 cell responses, indicating that adaptive IL-17 responses does not account for IL-17-mediated protection. Instead, we demonstrate that γ -PN vaccination promotes innate $\gamma\delta$ T-cell-derived IL-17A ($\gamma\delta$ T17) responses upon live *S. pneumoniae* challenge. Overall, the present study illustrates the possibility of using intranasal vaccination with a non-adjuvanted γ -PN vaccine to induce serotype-independent protection against lethal pneumococcal pneumonia and sepsis.

MATERIALS AND METHODS

Bacterial strains and growth conditions

S. pneumoniae strains used in the study have been previously described [21]. D39 (serotype 2) EF3030 (serotype 19F) and P9 (serotype 6A) were grown statically at 37 °C in 5% CO₂ in serum broth (SB) to D_{600} of 0.18, as previously described [21]. Strain Rx1 (capsule-negative strain derived from parent strain D39) and its various isogenic mutant derivatives were grown statically in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY) at 37 °C in 5% CO₂ to D_{600} of 0.5, as previously described [22].

Construction of Rx1[PdT/ Δ LytA] vaccine

Genetic manipulation was performed on strain Rx1 to delete the autolysin gene (*lytA*) in-frame, and replace the pneumolysin gene (*ply*) with a derivative encoding PdT (a non-cytolytic toxoid with three amino acid substitutions: Asp³⁸⁵ → asparagine, Cys⁴²⁸ → glycine and Trp⁴³³ → phenylalanine), essentially as previously described [23]. All PCR primers used are listed in Supplementary Table S1. Firstly, an Rx1 Δ Ply strain was constructed by replacing the Rx1 *ply* open reading frame with an erythromycin resistance (*erm*^r) cassette by transformation [23]. The *erm*^r cassette contains the 5' and 3' flanking regions of *ply* fused to the 5' and 3' termini, respectively, of the *erm*^r gene. The 5' flanking region of *ply* was obtained by PCR using primers PlyFlankF and PlyEryX, whereas the 3' flanking region was obtained using primers PlyFlankR and PlyEryY. The *erm*^r gene was PCR amplified using primers J214 and J215. Overlap extension PCR [24] using the above three PCR products was then performed to generate the cassette, which was used to transform Rx1, generating Rx1 Δ Ply. The *erm*^r gene in this construct was then replaced with *pdT* by transformation with a PdT cassette comprising the PdT open reading frame flanked by *ply* 5' and 3' flanking regions. Assembly of this cassette involved PCR amplification of *ply* 5' and 3' flanking regions using PlyFlankF and PlyRevX, and PlyForY and PlyFlankR, respectively. The *pdT* gene was obtained by PCR amplification of a PdT-expressing derivative of *S. pneumoniae* D39 [25] using PdTcloneF and PdTcloneR. The three components of the cassette were then fused and amplified by overlap extension PCR and used to transform Rx1 Δ Ply, generating Rx1[PdT].

A similar strategy was then used to delete the *lytA* gene from Rx1[PdT]. First, a tagged *lytA* deletion mutant was generated by transformation with a cassette comprising a spectinomycin resistance gene (*spec*^r) fused to *lytA* 5' and 3' flanking regions. The 5' flanking region of *lytA* was obtained by PCR using primers

LytAflankF and LytAspecX, whereas the 3' flanking region was obtained using primers LytAflankR and LytAspecY. The *spec^r* (*aad9*) gene was PCR amplified using primers J253 and J254. The cassette was then assembled and amplified by overlap extension PCR and used to transform Rx1[PdT]. The *spec^r* gene was then removed from the resultant Rx1[PdT/ Δ LytA::*spec^r*] construct by transformation with an overlap extension PCR product that fused the *lytA* 5' and 3' flanking regions, thereby deleting the *lytA* gene in-frame. The 5' flanking region of *lytA* was obtained by PCR using primers LytAflankF and LytANullA, whereas the 3' region was obtained using primers LytAflankR and LytANullB. The two fragments were fused and amplified by overlap extension PCR using the two outer primers, and then used to transform Rx1[PdT/ Δ LytA::*spec^r*]. The final Rx1[PdT/ Δ LytA] construct was validated by PCR, DNA sequencing, Western blotting and a haemolysis assay.

Generation of the γ -irradiated Rx1[PdT/ Δ LytA] vaccine

The Rx1[PdT/ Δ LytA] vaccine strain was first grown in THY as described above, and centrifuged at $4000 \times g$ for 10 min at 4 °C to collect the cells, which were then resuspended in PBS with 10% glycerol at a density of 1×10^{10} colony forming units (CFU)/ml. Two hundred microlitre volumes were aliquoted into 1.5 ml cryovials and frozen at -80°C . Vials containing the concentrated Rx1[PdT/ Δ LytA] vaccine were despatched on dry ice to the Australian Nuclear Science and Technology Organization (ANSTO, Lucas Heights, NSW). The cells were inactivated by exposure to a ^{60}Co γ -irradiation source and received a dose of 12 kilo-Gray (kGy). The cells were kept frozen on dry ice during the γ -irradiation process. The resultant γ -irradiated Rx1[PdT/ Δ LytA] vaccine, designated ' γ -PN' was transported to the University of Adelaide on dry ice and stored at -80°C , until used. To confirm complete inactivation of cells, a sample of γ -PN was thawed, plated out on to blood agar plates and grown overnight at 37 °C in 5% CO_2 .

Western blotting

Western blotting was performed essentially as described previously [26]. The en-capsulated strain D39, the parent Rx1 strain and the vaccine strain Rx1[PdT/ Δ LytA] (pre-irradiation) were grown to approximately 2×10^8 CFU/ml and lysates from 20 ml aliquots were analysed. After the transfer, the membrane was probed with either mouse anti-Ply (1/1000 dilution) or mouse anti-LytA (1/2000 dilution) polyclonal antisera, which was detected with an IRDye 800CW goat anti-mouse IgG (LI-COR). The blot was visualized using the Odyssey imaging system.

Haemolysis assay

A haemolysis assay was used to confirm the haemolytic activity of pneumolysin as described previously [27]. The parent Rx1 strain, Rx1[ermR/ Δ LytA] and the vaccine strain Rx1[PdT/ Δ LytA] (pre-irradiation) were grown to approximately 2×10^8 CFU/ml. Three milliliters aliquots of the samples were French pressed at 12000 psi to obtain supernatants for use in the assay.

Ethics statement

Animal experimentation was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition [2004] and 8th edition [2013]) and the South Australian Animal Welfare Act 1985. The protocol was approved by the Animal Ethics Committee at The University of Adelaide (Approval numbers S-2010-001 and S-2013-053).

Intranasal sepsis model

Wild-type (WT) C57BL/6 mice (5–6 weeks old) were supplied by Laboratory Animal Services at The University of Adelaide, South Australia. μ MT mice (5–6 weeks old) were originally obtained from H. Korner (Menzies Institute for Medical Research, University of Tasmania, Australia) and bred in-house at the University of Adelaide Animal Facility. Mice were first anaesthetized intraperitoneally (IP) with pentobarbital sodium (Nembutal; Ilium) at a dose of $66 \mu\text{g/g}$ of body weight. The anaesthetized mice then received the γ -PN vaccine by gently applying $30 \mu\text{l}$ ($\sim 1 \times 10^8$ CFU equivalents per dose) intranasally (IN) to the nostrils. Control mice received PBS. Mice were immunized IN twice at 2-week intervals. Sera were collected from the mice by sub-mandibular bleeding 1 week after the last immunization. A week later thereafter, mice were challenged IN (as described above) with strains D39 (1×10^6 CFU in $30 \mu\text{l}$) or P9 (5×10^6 CFU in $30 \mu\text{l}$). Infected mice were then monitored for up to 21 days to determine the number of days until they were moribund.

Intranasal pneumonia model

For the pneumonia model, immunization and challenge procedures were essentially the same as described above, except that mice were challenged IN with strain EF3030 (1×10^7 CFU in $30 \mu\text{l}$). Four days post challenge, mice were euthanized and the nasal wash and homogenized nasopharyngeal tissue and lung tissue were plated for CFU recovery as described previously [28]. Total CFU in the nasopharynx includes the CFU from the nasal wash and nasal tissue combined.

Intranasal colonization model

For the colonization model, un-anaesthetized C57BL/6 mice were infected with EF3030 by gently restraining the mice and applying $10 \mu\text{l}$ (1×10^7 CFU) to the nostrils. Seven days later, mice were euthanized and the nasal wash and nasopharyngeal tissue were harvested and plated for CFU recovery as described above.

IFN γ /IL-17A neutralization *in vivo*

Two weeks after mice received the second dose of γ -PN vaccine as described above, they were treated with neutralizing monoclonal antibodies (all from BioXCell) to IL-17A or interferon- γ (IFN- γ) [29]. Mice were administered three IP doses ($200 \mu\text{g}$ in $200 \mu\text{l}$ PBS per dose) of either anti-IL-17 (17F3), anti-mouse IgG1 (MOPC21) (isotype control for 17F3), anti-IFN- γ (XMG1.2) or anti-rat IgG1 (HRPN) (isotype control for XMG1.2) at day -1 (24 h before the challenge), day 0 (6 h post challenge) and day 2 (48 h post challenge). Mice were challenged IN with D39 as described above and monitored for up to 21 days to determine the number of days until they were moribund.

Measurement of antibody responses

Sera collected from immunized mice were assayed by ELISA to determine pneumococcal surface protein A (PspA)-specific antibody responses as described previously [21]. End point titres are expressed as the reciprocal of the last dilution whose OD value was equal to or more than the cut-off value. The cut-off value was determined by adding 3-fold S.D. to the mean (i.e. mean + 3 S.D.) of the OD values of samples from PBS infected mice (negative controls to determine background levels). We used alkaline phosphatase (AP) conjugated rabbit anti-mouse IgG (Invitrogen) and goat anti-mouse IgA (Zymed) as detecting antibodies.

Splenocyte stimulation

Spleens were harvested 2 weeks after the second IN immunization with γ -PN vaccine and were used for splenocyte stimulation as described previously [30] with a few modifications. To obtain cell suspensions, splenocytes were passed through a 70 μ m mesh strainer (BD) and suspended in complete RPMI 1640 (RPMI 1640 with Phenol Red supplemented with 10% foetal calf serum, 1% L-glutamine, 1% streptomycin/penicillin and 0.2% 2-mercaptoethanol). Red blood cells were then lysed using red blood cell lysis buffer. Splenocytes were plated into 96 well tissue culture plates at a concentration of $\sim 1.8 \times 10^6$ cells in 200 μ l. Splenocytes were stimulated with 50 μ l of complete RPMI containing 1×10^6 CFU/ml of γ -PN or MalX antigen (10 μ g/ml). Complete RPMI 1640 medium alone served as a control. Seventy-two hours later, cells were collected following centrifugation and used for intracellular cytokine staining.

Lung digestion to retrieve lymphocytes for flow cytometric analysis

Lungs were collected from immunized mice at 24 and 48 h post challenge. The lungs were perfused with ice-cold PBS and subsequently dissected and placed in 1 ml digestion medium [Dulbecco's modified Eagle medium (DMEM) containing sodium pyruvate (Thermo Fisher Scientific) supplemented with 1% HEPES, 5% FCS, 1% penicillin/gentamicin, 2.5 mM calcium chloride, collagenase D (Roche; 1 mg/ml) and DNase (Roche; 40 units/ml)]. Lungs were then finely macerated with surgical scissors and incubated at 37°C for 1 h (with mixing every 20 min). Samples were then processed to obtain single cell suspensions as described above and subjected to intracellular cytokine staining.

Intracellular cytokine staining and flow cytometry

Lung cell suspensions or splenocytes stimulated with γ -PN were used for intracellular cytokine staining using the protocol described previously [31]. For stains involving Foxp3 detection, cells were permeabilized using the Foxp3/transcription factor staining buffer set (eBioscience), whereas for all other stains the Cytotfix/Cytoperm kit (BD) was used, according to respective manufacturers' instructions. Cell surface antigens were stained using the following antibodies: anti-CD3 (APC; Biolegend), anti-CD4 (BV450; eBioscience), anti-CD44 (PE-Cy7, eBioscience), anti-CD44 (FITC, BD), anti-CD103 (PE, eBioscience), anti-

CD11a (APC, eBioscience), anti- $\gamma\delta$ TCR (PercPcy5.5, Biolegend or PECy7, Biolegend), anti-F4/80 (FITC, BD), anti-Gr1 (PE, BD), anti-CD11b (PECy7, BD) and anti-CD45.2 (Biotin, BD). The following intracellular antibodies were used: anti-IL-17A (BV510, Biolegend), anti-IFN- γ (FITC, eBioscience), anti-IFN- γ (PECy7, eBioscience), anti-IL-4 (PE, eBioscience) and anti-Foxp3 (PerCPCy5.5, eBioscience). Biotin-conjugated antibodies were detected using Streptavidin-BV450 (BD). In all cases, dead cells were excluded from analyses using LIVE/DEAD fixable near-infrared dye (Molecular Probes). Cells were acquired using an LSRII flow cytometer (BD) and data were analysed using FlowJo software (TreeStar).

Statistics

Data were analysed using a two-tailed Fisher's exact test, unpaired Student's *t* test or a Mann-Whitney *U* test, as appropriate. Results with *P*-values less than 0.05 were considered statistically significant. Statistical analysis was performed using Graph Pad Prism.

RESULTS

Characterization of the γ -PN vaccine

The γ -PN vaccine (Rx1[PdT/ Δ LytA]) was generated by replacing the *ply* gene in *S. pneumoniae* Rx1 with a derivative encoding PdT, and then deleting the *lytA* gene. PdT is a toxoid derivative of Ply carrying three amino acid substitutions (Asp³⁸⁵ \rightarrow asparagine, Cys⁴²⁸ \rightarrow glycine and Trp⁴³³ \rightarrow phenylalanine) that abrogate both the complement activation and cytotoxic properties of the toxin, without affecting immunogenicity [25,32]. The purpose of deleting *lytA* was to facilitate large scale culture to high cell densities *in vitro*, by preventing onset of cellular autolysis in stationary phase. Expression of PdT and deletion of *lytA* were confirmed by Western blot analysis (Figure 1A). Anti-Ply and anti-LytA antibodies detected both LytA and native Ply in D39 (lane 2), the parent strain (Rx1) (lane 3) and the respective purified control proteins (lane 1). As expected, no LytA related bands were detected in lysates of pre-irradiated vaccine strain Rx1[PdT/ Δ LytA] samples (lane 4). In contrast, bands related to Ply were still detectable. Complete absence of haemolytic activity of the final vaccine strain Rx1[PdT/ Δ LytA] (pre-irradiation) was also confirmed by a haemolysis assay (Figure 1B). Furthermore, the absence of antibiotic resistant genes in the vaccine strain was confirmed using both gene sequencing and plating on media containing the relevant antibiotic (results not shown).

Immunization with γ -PN provides serotype-independent protection

Considering that γ -PN is derived from the un-encapsulated strain Rx1, we initially investigated whether the vaccine was capable of providing protection against IN challenge by the parental encapsulated (type 2) strain D39. Our data show that intranasal vaccination of mice with γ -PN resulted in a statistically significant increase in survival rate (*P* = 0.0201) compared with mock-immunized control mice following lethal challenge with *S.*

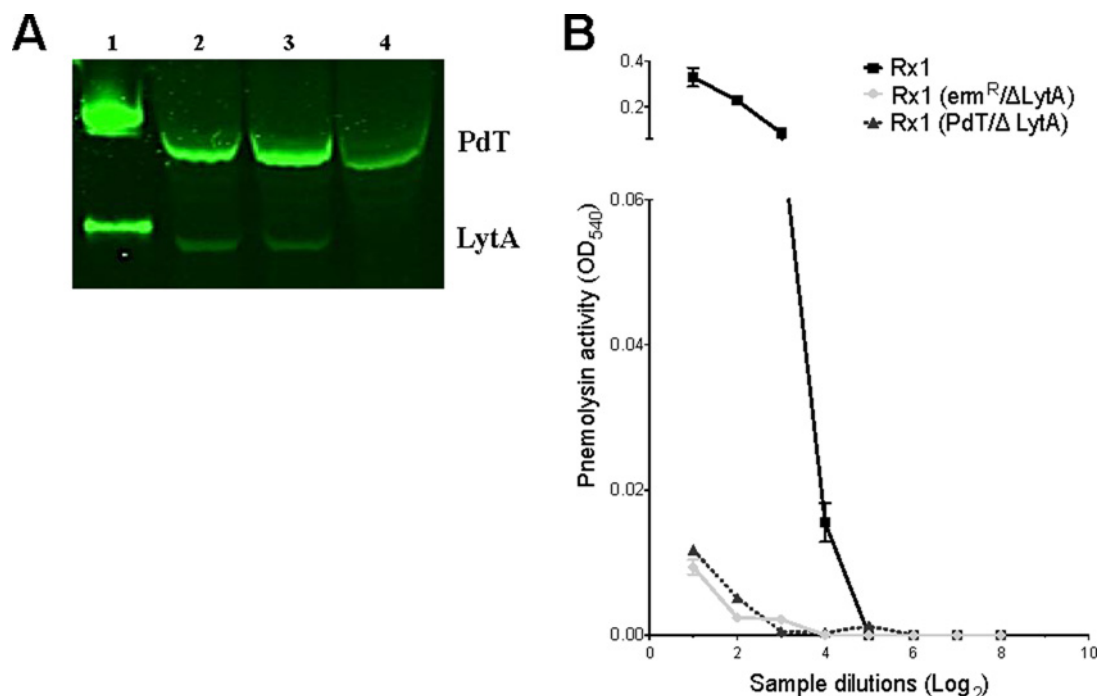


Figure 1 Expression of PdT and LytA

(A) Expression of Ply/PdT (53 kDa) and LytA (36 kDa) was determined by Western blotting, as described in 'Materials and Methods' section. Lanes: 1, purified Ply and LytA (5 ng each); 2, *S. pneumoniae* D39; 3, *S. pneumoniae* Rx1; 4, Rx1[PdT/ΔLytA] vaccine strain (pre-irradiation). The recombinant purified Ply and LytA proteins are larger in size due to the presence of a His-tag. (B) Ply cytotoxic activity in lysates of the indicated strains (2×10^8 CFU/ml) was determined by haemolysis assay.

pneumoniae D39 (Figure 2A). To investigate whether the vaccine can provide serotype-independent protection, we challenged γ -PN immunized mice with *S. pneumoniae* P9 (serotype 6A). Similarly, IN vaccination with γ -PN afforded significant protection against P9 challenge ($P = 0.0431$; Figure 2B), suggesting that the γ -PN vaccine is capable of providing both serotype-dependent and -independent protection against lethal pneumococcal challenge.

In addition, we examined whether the γ -PN vaccine could provide protection against local infection in the lung. Immunized mice were challenged under anaesthesia with EF3030 (serotype 19F), which causes focal pneumonia but not lethal sepsis [33,34] and bacterial counts in the lungs and nasopharynx were determined 4 days post challenge. Our data show that vaccination with γ -PN reduced bacterial counts in the lungs ($P = 0.0397$) when compared with control mice (Figure 2C). Although median CFU in the nasopharynx was also slightly lower in γ -PN vaccinated mice, this did not reach statistical significance (Figure 2C). To further explore whether effects on colonization might contribute to vaccine efficacy, we utilized a colonization model. Vaccinated and control mice were inoculated IN with a lower dose of *S. pneumoniae* EF3030 without anaesthesia and bacterial counts were determined in the nasopharynx at day 7 post inoculation. Our data indicate that vaccination with γ -PN did not affect nasopharyngeal colonization with EF3030, with a similar number of CFU in the nasopharynx of both vaccinated and control mice (Figure 2D).

γ -PN efficacy is dependent on B-cell responses

To elucidate the underlying mechanisms of γ -PN-mediated protection, we examined the role of B-cell responses in acquired immunity to *S. pneumoniae*. Induction of antibody-initiated complement-dependent killing represents the hosts' major defence mechanism against pneumococcal challenge [1], and generally current approaches for pneumococcal vaccines rely on the efficacy of B-cell responses [8]. Thus, to evaluate the ability of γ -PN to induce antigen-specific antibody responses, we assayed IgA and IgG responses to PspA, a member of the choline-binding protein family of *S. pneumoniae* surface proteins, and a proven protective immunogen [35,36]. Significant levels of PspA-specific IgA ($P = 0.0002$) and IgG ($P = 0.0241$) were detected in sera collected from immunized mice 14 days after the second vaccination, relative to those in PBS treated controls (Figure 3A).

Next, to determine whether the vaccine's efficacy is dependent on B-cell responses, μ MT mice (B-cell-deficient mice) and WT (C57BL/6) mice were immunized IN with γ -PN and challenged IN with D39, and animals were monitored for a period of 21 days. As shown in Figure 3B, γ -PN-vaccinated WT mice show significant protection against lethal D39 challenge compared with PBS treated controls, as indicated by a significantly greater survival rate ($P = 0.0059$). In contrast, γ -PN-immunized B-cell-deficient μ MT mice did not show protection when compared with PBS treated μ MT control mice (Figure 3B). This suggests that the protective efficacy of the γ -PN vaccine against sepsis is reliant on B-cell responses.

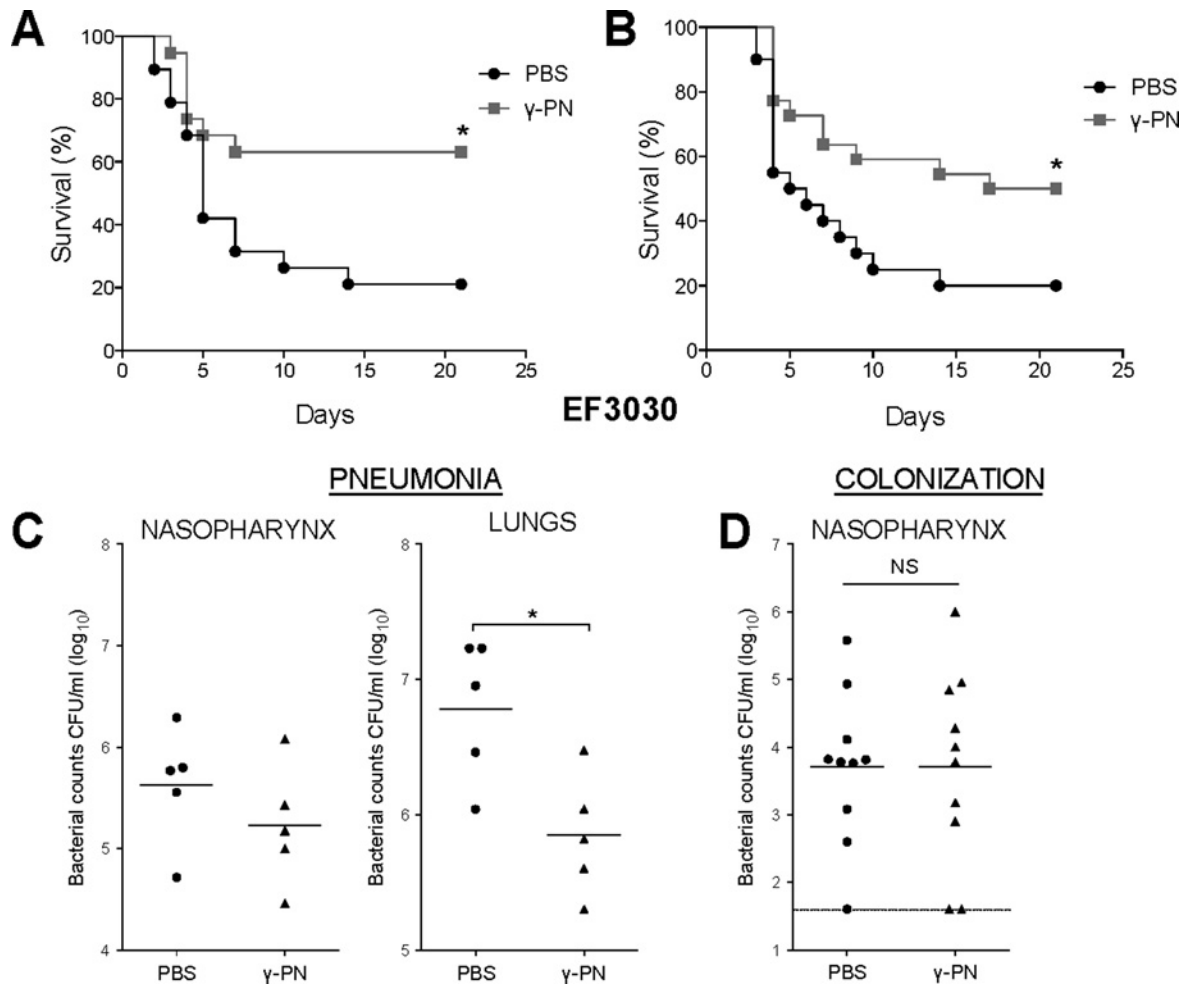


Figure 2 Protection against sepsis and focal pneumonia elicited by immunization with γ -PN

Mice were immunized IN with two doses of γ -PN or given PBS. Two weeks after the second immunization, mice ($n = 20$ per group, mice pooled from two independent experiments of $n = 10$) were challenged IN under anaesthesia with *S. pneumoniae* D39 (A) or P9 (B). Survival curves are shown and overall survival rate was determined at 21 days. Data were analysed using Fisher's exact test ($*P < 0.05$). Alternatively, immunized or control mice were challenged with (pneumonia model) or without (colonization model) anaesthesia with *S. pneumoniae* EF3030. For the pneumonia model, bacterial counts in the lungs and nasopharynx 4 days following challenge are shown (C). For the colonization model, bacterial counts in the nasopharynx at 7 days post inoculation are shown (D). Differences in bacterial load were analysed using the Mann-Whitney U test ($*P < 0.05$). NS, not significant.

IL-17 plays an essential role in the protective efficacy of γ -PN

In addition to B-cell responses, previous studies have demonstrated that both IFN- γ and IL-17 are important components of host defences against *S. pneumoniae* [30,37–40]. Therefore, we investigated whether either of these cytokines contributed to the protective efficacy of the γ -PN vaccine. Mice immunized IN with γ -PN were treated with three doses of neutralizing antibodies to IL-17 or IFN- γ , or isotype control antibodies, at days -1 , 0 and 2 post challenge with a lethal dose of D39 and were then monitored for survival (Figure 4). Administration of isotype controls for both IFN- γ (Figure 4A) and IL-17 (Figure 4B) did not alter the protective efficacy of γ -PN against D39 challenge

($P = 0.0354$ for anti-IFN- γ isotype control group, $P = 0.0018$ for anti-IL-17A isotype control group). Importantly, γ -PN induced significant protection in immunized mice despite IFN- γ neutralization ($P = 0.0083$) when compared with their relative control (Figure 4A). However, the reduced survival rate of non-immunized mice in the IFN- γ treated group compared with the isotype control group was expected due to its known role in primary host defence against pneumococcal challenge [37,38]. In contrast with IFN- γ neutralization, γ -PN-induced protection was abolished by IL-17A neutralization (Figure 4B). Surprisingly, IL-17A neutralization did not influence the lethality rate of pneumococcal infection in non-immunized mice, which illustrates the role of IL-17A in the secondary immune response.

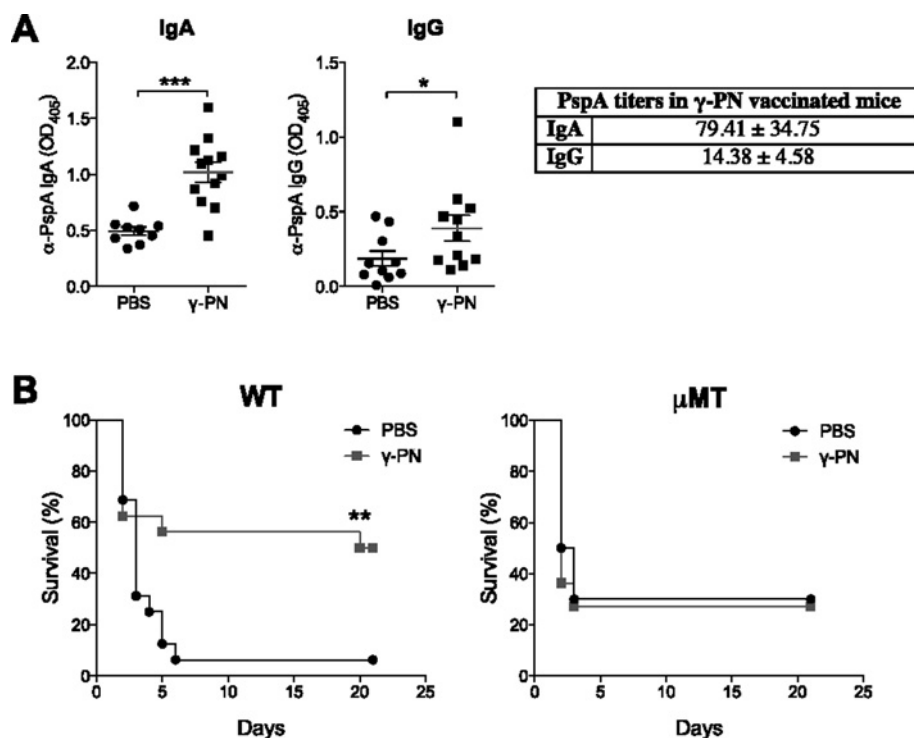


Figure 3 The efficacy of the γ -PN vaccine is dependent on B-cell responses

(A) Sera were collected 2 weeks after the second immunization dose with γ -PN and assayed for PspA-specific IgA and IgG antibodies by ELISA. Antibody titres are represented as mean \pm S.E.M. ($n = 11$). End point titres are expressed as the reciprocal of the last dilution with OD value equal to or more than the cut-off value. The cut-off value was determined by adding 3-fold S.D. to the mean (i.e. mean + 3 S.D.) of the OD values of samples from PBS-treated mice. (B) WT or μ MT mice ($n = 10$ minimum per group) were immunized IN with two doses of γ -PN or PBS, and 2 weeks after the second immunization, mice were challenged IN with D39. Mice were monitored for survival for 21 days post challenge. Differences in survival rate between immunized and control mice were analysed by Fisher's exact test (** $P < 0.01$).

These data demonstrate that the mechanisms of protection elicited by γ -PN immunization are IL-17A dependent and in part IFN- γ dependent.

Vaccination with γ -PN induces CD4⁺-independent IL-17 immunity

In general, IL-17 derived from Th17 cells has been reported to be the essential component of antigen-specific memory T-cell responses during pneumococcal infections, particularly for driving the recruitment of macrophages and neutrophils into the nasopharynx to mediate clearance [40]. Th17-driven immunity in anti-pneumococcal vaccination strategies has also been clearly demonstrated, as neutralization of IL-17 was reported to interfere with protection against colonization mediated by mucosal immunization with a whole-cell vaccine [30]. Considering the IL-17-dependent protective responses induced by γ -PN (Figure 4), we next investigated the mechanism underlying γ -PN-induced IL-17-dependent immunity and examined whether γ -PN induces antigen-specific Th17 cells. To do this, splenocytes were harvested from immunized and control mice and stimulated *ex vivo* with the γ -PN vaccine or a proven Th17 protective antigen MalX [10] for 72 h. Following stimulation, cells were subjected to intracellular cytokine staining for analysis of T helper cell subsets (Th17, Th1, Th2, Treg). Our data show that stimulation of splenocytes from γ -PN immunized mice did not induce a significant increase

in the percentage of Th17, Th1, Th2 or Treg relative to the PBS control following stimulation with either the γ -PN vaccine or MalX antigen (Figure 5B). These data suggest that γ -PN does not induce circulating Th17 memory cell responses.

To further investigate the memory responses induced by γ -PN vaccine, we analysed whether IL-17 dependency could be related to tissue resident memory (TRM) cells. TRM cells are a unique memory subset as they reside in sites of infection after initial pathogen clearance, provide the first line of antigen-specific defence upon re-infection, and elicit heightened protection relative to circulating central memory cells [41,42]. Analysis of lung tissue from γ -PN immunized mice showed that these mice did not induce CD4⁺ TRM cells [TRM total, gated CD44^{hi}CD103⁺ and were also CD11a^{hi} (results not shown)] relative to the PBS control, nor were there differences in the ability of these cells to make IL-17A (TRM IL-17⁺; Figure 5C). Collectively, these data indicate that vaccination with γ -PN does not elicit CD4⁺ TRM cell responses.

Considering that we did not detect a significant increase in Th17 or TRM IL-17⁺ cells, we investigated the possible role of IL-17-producing innate immune cells in γ -PN vaccinated animals. In addition to Th17 cells, non-conventional T-cells such as $\gamma\delta$ [43] and NKT cells [44] located in mucosa have been shown to elicit protective pulmonary IL-17A responses. In particular, $\gamma\delta$ T17 has been shown to strongly promote the recruitment of

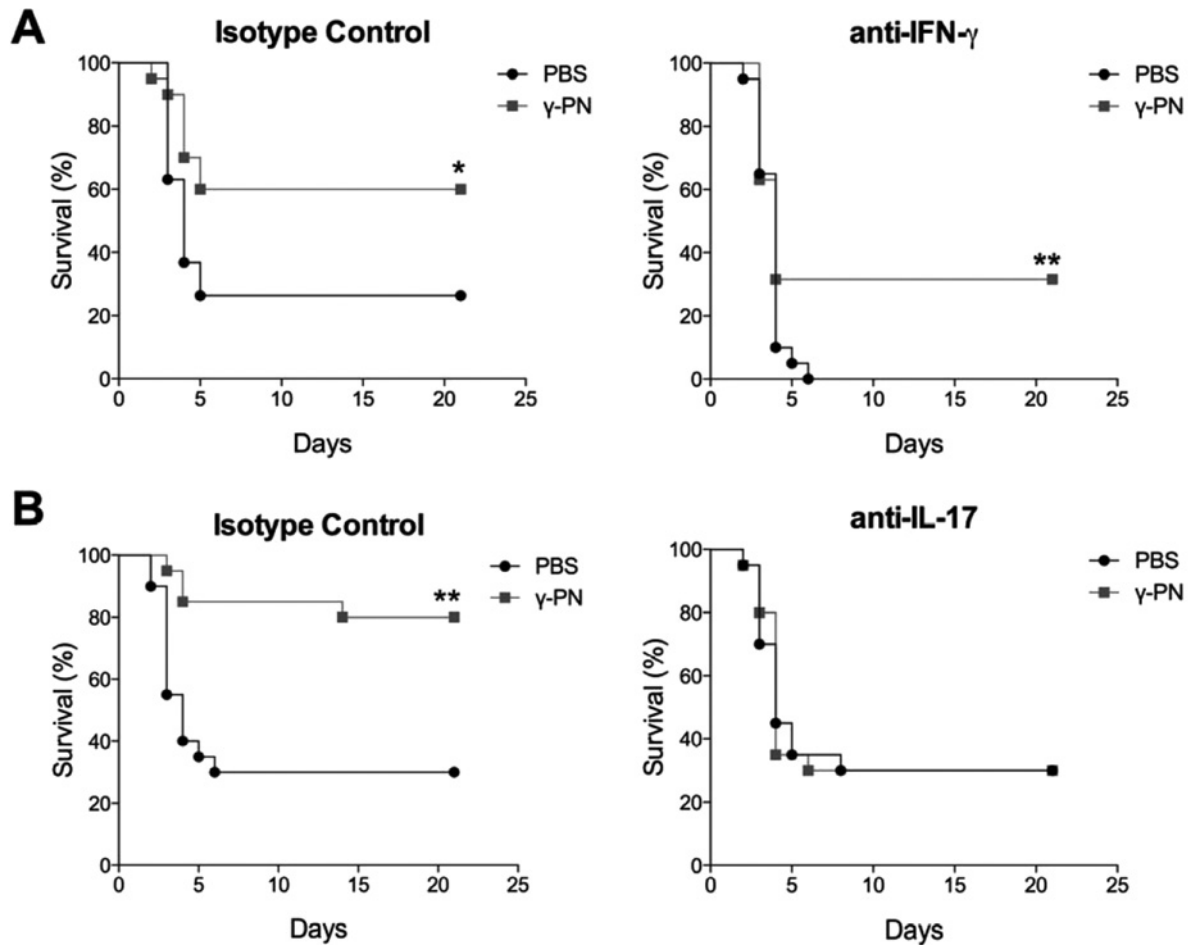


Figure 4 Depletion of IL-17, but not IFN- γ abrogates protection induced by γ -PN

Mice were immunized IN with two doses of γ -PN or given PBS and 2 weeks after the second immunization, mice ($n =$ minimum 20 per group, mice pooled from three independent experiments) were injected IP with 200 μ g of anti-IFN- γ (A), anti-IL-17 (B) or the respective isotype control, on day -1 (24 h before challenge), day 0 (6 h post challenge with D39) and day 2 (48 h post challenge). Mice were monitored for survival for 21 days following challenge IN with D39. Differences in survival rates between groups of mice treated with cytokine-neutralizing and control antibodies were analysed by Fisher's exact test: * $P < 0.05$; ** $P < 0.01$.

neutrophils during pneumococcal infection [45]. Therefore, we hypothesized that the IL-17A-dependent immunity elicited by γ -PN may be due to heightened $\gamma\delta$ T17 responses. To investigate this, we examined $\gamma\delta$ T-cells and T effector cell numbers in the lungs of γ -PN immunized mice 24 and 48 h post D39 challenge. γ -PN vaccination did not alter the total number of T effector cells or the relative populations of Th1 or Th17 cells in the lung 24 h post D39 challenge (Figure 6A). Similar results were observed at 48 h for total T effector cells and Th17. However, we detected a significant decrease in Th1 cell numbers in γ -PN-immunized mice ($P = 0.0452$) relative to the PBS-treated controls at 48 h post challenge. In contrast with T effector cells, IN vaccination with γ -PN induced significant changes in $\gamma\delta$ T-cell populations in the lung post D39 challenge (Figure 6B). Although the total number of $\gamma\delta$ T-cells in the lung of vaccinated mice was not significantly different relative to non-immunized mice, our data indicate that IN vaccination with γ -PN specifically enhanced $\gamma\delta$ T17 cell numbers in the lungs at 24 h ($P = 0.0297$) and this was

further enhanced at 48 h post challenge ($P = 0.0157$). Interestingly, we detected a decrease in $\gamma\delta$ T1 cell numbers in vaccinated mice at 24 h, leading to a significant difference ($P = 0.0267$) detected at 48 h relative to the control animals. Overall, these data demonstrate that γ -PN promotes a significant increase in $\gamma\delta$ T17 cell responses associated with a significant decrease in Th1 and $\gamma\delta$ T1 cells and no difference in the Th17 population. Therefore, our data suggest that $\gamma\delta$ T17 cells may be a potential source for IL-17A involved in mediating protective immunity in γ -PN-vaccinated mice.

Given that IL-17 is known to have a prominent role in driving the influx of phagocytic cells, we investigated the relative numbers of neutrophils and macrophages in the lungs of γ -PN-immunized mice at 24 and 48 h post D39 challenge (Figure 6C). Importantly, the numbers of either cell type remains similar at 24 and 48 h post challenge in γ -PN-immunized mice. In contrast, we detected a significant increase in macrophages in the lungs of PBS control mice between 24 and 48 h post challenge

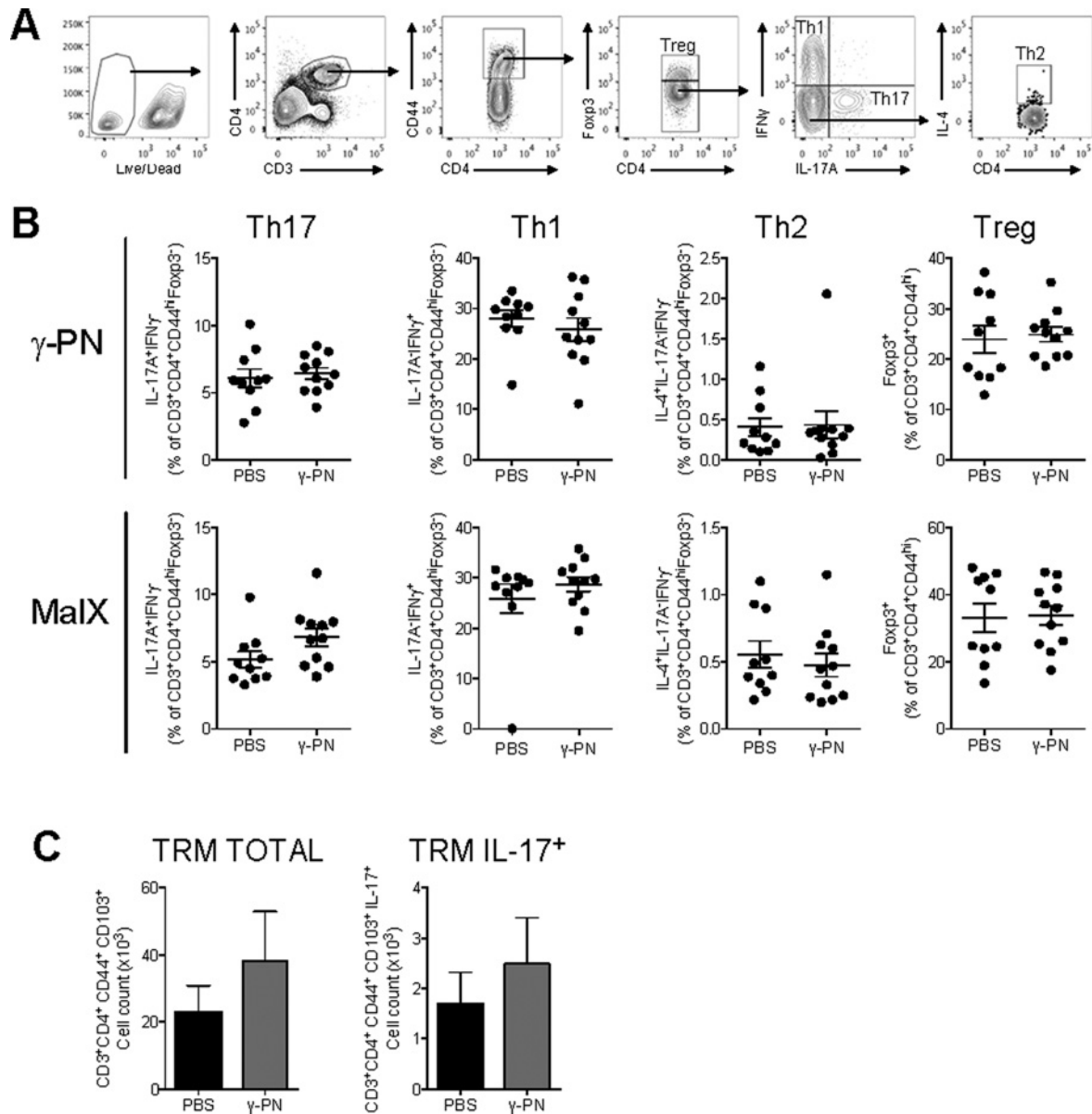


Figure 5 γ -PN does not induce antigen-specific Th17 cells or tissue-resident memory CD4⁺ T-cells

Mice were immunized IN with two doses of the γ -PN vaccine and 2 weeks after the second immunization, the spleens were harvested and restimulated in culture with γ -PN or media alone (negative control) for 72 h. (A) Representative flow cytometric data scheme for analysis of T helper cell subsets on cultured splenocytes 72 h following stimulation: Th1 (IFN- γ ⁺ IL-17A⁻), Th17 (IL-17⁺IFN- γ ⁻), Th2 (IL-17⁻IFN- γ ⁻IL-4⁺) and Treg (Foxp3⁺). (B) Percentage of T-cells following stimulation with the γ -PN vaccine or MalX antigen using flow cytometry. (C) Mice were immunized IN with two doses of γ -PN and 2 weeks after the second immunization, mice were challenged with D39 and the lungs were harvested and analysed for the total number of CD4⁺ TRM cells (CD3⁺CD4⁺CD44⁺CD103⁺) and CD4⁺ TRM IL-17⁺ cells (CD3⁺CD4⁺CD44⁺CD103⁺) by flow cytometry. Results are presented as mean \pm S.E.M. and representative of two independent experiments ($n = 10$).

($P = 0.0122$). This trend was also apparent for neutrophils although it did not reach statistical significance.

DISCUSSION

In recent years there has been a considerable focus on developing pneumococcal vaccines that are serotype independent, cost ef-

fective and capable of providing protective immunity in people of all ages. The success of using γ -irradiation to inactivate bacteria has been demonstrated for *Rickettsia* [46], *Brucella abortus* [20] and *Listeria monocytogenes* (Lm) [14]. In the present study, we administered γ -irradiated, un-encapsulated pneumococcal whole cells to maximize exposure of clinically relevant antigens in their native form. Moreover, the intranasal route of immunization was chosen to reflect the natural route of exposure to

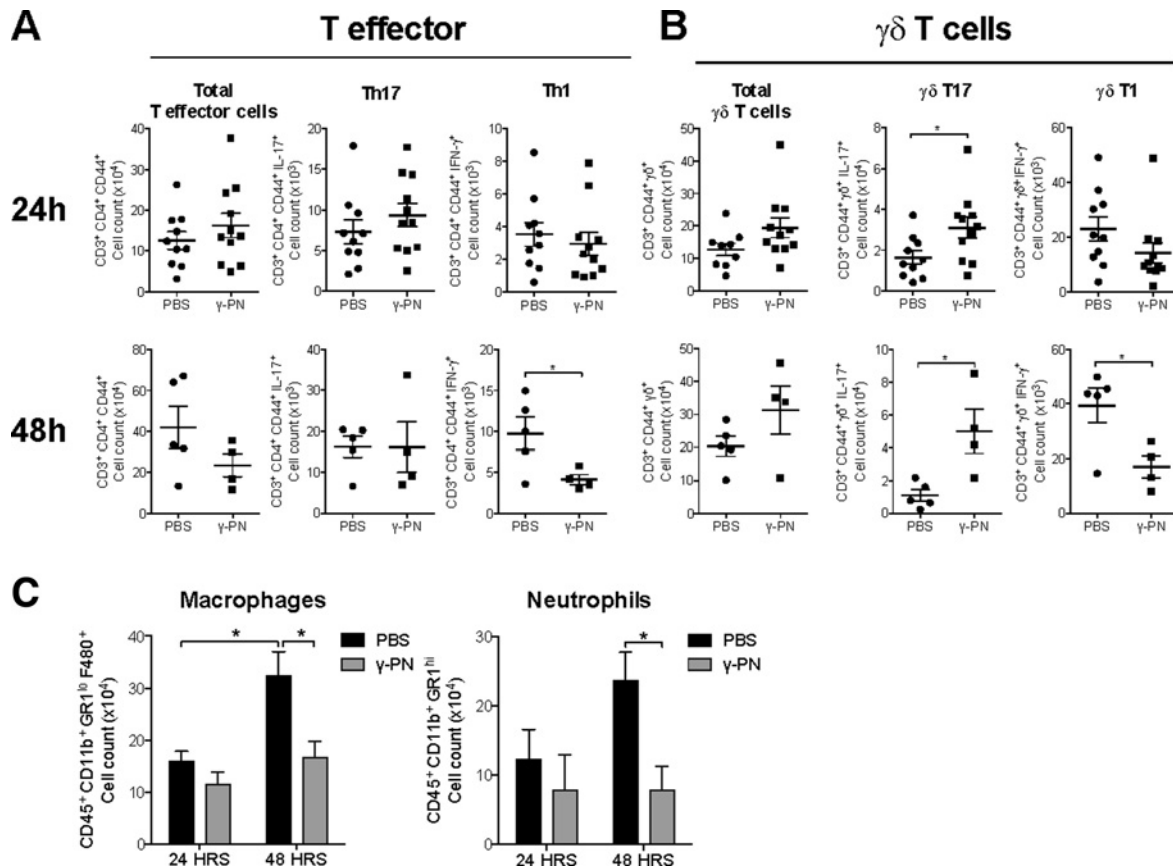


Figure 6 Vaccination with γ -PN induces IL-17 via T cells, not CD4⁺ T cells

Mice were immunized IN with two doses of γ -PN or given PBS, and 2 weeks after the second immunization, mice were challenged IN with D39. Lungs were harvested at 24 and 48 h post challenge and analysed for the total number of the following cells by flow cytometry. **(A)** T effector cells: total T effector cells (CD3⁺CD4⁺CD44⁺), Th17 (CD3⁺CD4⁺CD44⁺IL-17⁺), Th1 (CD3⁺CD4⁺CD44⁺IFN- γ ⁺); **(B)** $\gamma\delta$ T-cells: total $\gamma\delta$ T-cells (CD3⁺CD44⁺ $\gamma\delta$ TCR⁺), $\gamma\delta$ T1 (CD3⁺CD44⁺ $\gamma\delta$ TCR⁺IFN- γ ⁺), $\gamma\delta$ T17 (CD3⁺CD44⁺ $\gamma\delta$ TCR⁺IL-17⁺); and **(C)** phagocytic cells: macrophages (CD45⁺CD11b⁺Gr1^{lo/-}F4/80⁺) and neutrophils (CD45⁺CD11b⁺Gr1^{hi}). Results are presented as mean \pm S.E.M.; * P < 0.05 (Student's t test). Results are representative of two independent experiments (n = 10) for 24 h and (n = minimum 4) for 48 h time point.

pneumococcus in humans. During the initial stages of pneumococcal colonization of the nasopharynx, the CPS is down-regulated and there is increased exposure of cell wall teichoic acids and surface proteins, which facilitate attachment to the mucosa [47,48]. Thus, intranasal delivery of an un-encapsulated whole-cell vaccine may elicit enhanced immunity towards these sub-capsular structures. γ -Irradiation is also a superior inactivation technique, as it maintains cellular structures and prevents disruption of antigens, which can often occur with alternative heat inactivation methods as shown with Lm [14] and chemical or UV inactivation methods as shown for influenza [49]. We have shown that intranasal immunization with γ -PN provides significant serotype-independent protection against sepsis caused by D39 and P9 and focal pneumonia caused by EF3030.

To investigate the underlying mechanism for serotype-independent protection induced by γ -PN, we evaluated the role of B- and T-cells in acquired immunity. Lack of protective immunity in vaccinated μ MT mice illustrates the significant contribution of B-cell responses. Generally, antibodies against capsular and

sub-capsular antigens are important for complement fixation and opsonization in systemic clearance of pneumococci [1]. Thus, our data suggest that humoral immunity induced by γ -PN is critical for the protection observed against sepsis and pneumococcal pneumonia. Furthermore, analysis of sera from γ -PN-immunized mice showed induction of PspA-specific IgG and IgA responses. Humoral responses against PspA are crucial to prevent interference with complement-mediated opsonization, one of PspA's major roles in pneumococcal pathogenesis [1]. Humoral immunity has also been implicated in defence against colonization, although increasing evidence suggests that cellular immunity is more crucial [50]. Indeed, Th17 cell responses have been shown to be important for abrogation of the initial stages of bacterial colonization [30,51,52], and the induction of a Th17 response has also been shown to be an important component for immunity induced by a cholera-toxin adjuvanted whole-cell-based pneumococcal vaccine [30]. In the present study, we showed that IN vaccination with γ -PN did not induce antigen-specific Th17 responses, which is a probable explanation for why we did not observe protection in the EF3030 colonization model. Nevertheless, neutralization

experiments showed that IL-17 is important for γ -PN vaccine efficacy.

Interestingly, analysis of lymphocytes from the lungs following D39 challenge revealed that γ -PN immunization resulted in enhanced levels of pulmonary $\gamma\delta$ T17 cells. These cells represent one of the major innate sources of IL-17A during infection, which plays critical roles in protection against extracellular pathogens such as *S. pneumoniae*. In addition, $\gamma\delta$ T-cell-deficient mice have low survival rates that were shown to be associated with higher bacterial loads and reduced neutrophilia following pneumococcal infection [45,53]. IL-17 has also been shown to be crucial for the recruitment of neutrophils and macrophages as part of the host's defence against *S. pneumoniae* [40]. Therefore, it is feasible that γ -PN immunization may promote neutrophilia through enhanced $\gamma\delta$ T17 responses, which combines with opsonizing antibodies to clear pneumococcal infection. Furthermore, although we have shown a clear and prominent role for IL-17 in our vaccine-elicited protection, it also appears that IFN- γ may be playing a more supplementary role, which could explain the decrease in survival rates observed in IFN- γ neutralization studies. Similar to IL-17, IFN- γ has also been found to be involved in the induction of neutrophil chemoattractants to promote neutrophil influx to facilitate protection against *S. pneumoniae* [37]. Our data indicate that IFN- γ plays a more prominent protective role compared with IL-17 during primary infection.

Nevertheless, although our data illustrate the significantly increased number of $\gamma\delta$ T17 cells in the lung of vaccinated animals, other innate IL-17A producers such as NKT cells may also be involved. An emerging concept in $\gamma\delta$ T-cell biology is the ability of these cells to adopt 'memory'-like properties. Oral Lm infection induces a subset of protective $\gamma\delta$ T-cells that preferentially expand upon oral, but not intravenous Lm re-infection or oral *Salmonella* infection [54]. Similar observations have also been made in a model of *Staphylococcus aureus* infection [55]. Furthermore, in imiquimod-induced psoriasis inflammation, $\gamma\delta$ T17 cells have been shown to redistribute into non-inflamed skin tissue, are activated more rapidly via IL-1 β and accelerate secondary skin inflammation upon re-exposure to imiquimod [56]. In the present study, it is not clear whether γ -PN directly induces 'memory'-like $\gamma\delta$ T17 cell responses or whether there is an existence of a γ -PN-induced adaptive response that leads to enhanced $\gamma\delta$ T17 recruitment or expansion/sensitivity within the lung. More detailed studies will be required to elucidate the mechanisms of γ -PN-induced $\gamma\delta$ T17 cell responses.

Furthermore, immunological analysis of phagocytic cells at 24 and 48 h post D39 challenge showed no difference in total levels of macrophages and neutrophils in γ -PN immunized mice. The stable level of inflammation observed in vaccinated mice could imply that the majority of pneumococci are cleared within 48 h, compared with the increased inflammatory responses in control mice illustrated by the significant increase in phagocytic cells. In general, strong neutrophil influx during pneumococcal infection has been shown to be detrimental, with increased mortality rates in mice [57]. Therefore, rapid bacterial clearance and reduced influx of phagocytic cells may underpin the efficacy of γ -PN vaccine.

In addition, we detected a greater increase in the number of $\gamma\delta$ T17 cells in γ -PN-immunized mice at 48 compared with 24 h post challenge. This suggests that $\gamma\delta$ -derived IL-17 could be playing another role in the control of pneumococcal infection apart from promoting neutrophilia. Indeed, IL-17 has been shown to regulate the production of antimicrobial peptides such as β -defensin 2 [58], which has been reported to work synergistically with lysozymes to inhibit the growth of *S. pneumoniae* [59]. Thus, IL-17 induced by γ -PN may have also enhanced innate anti-microbial peptide defences against *S. pneumoniae*. In addition, it has been suggested that promoting IL-17 responses could be beneficial for alleviating the problem of super-infection that occurs between influenza and *S. pneumoniae*. In particular, IFN-I induced by influenza virus has been reported to compromise IL-17 production by $\gamma\delta$ T-cells, thereby exacerbating infection [60]. Therefore, γ -PN-induced $\gamma\delta$ T17 cells could also be advantageous in preventing the harmful effects of co-infection with other pathogens. Further experiments are required to fully elucidate the role and significance of γ -PN vaccine-induced IL-17 responses.

Our data also illustrate limited efficacy for γ -PN against nasopharyngeal carriage compared with previous reports using an ethanol-whole-cell pneumococcal vaccine that demonstrated protective efficacy against pneumococcal carriage via antibody independent, Th17-driven immunity [30]. However, experience with pneumococcal conjugate vaccines has shown that elimination of carriage may be a 'double edged sword', whereby the benefits of blocking transmission of strains covered by the vaccine can be offset by facilitating the phenomenon of 'serotype replacement' with other types of pneumococci, or indeed by promoting carriage of other significant bacterial pathogens such as *S. aureus* [61]. An important additional consideration is that efficacy of previous experimental pneumococcal vaccines targeted for mucosal administration, such as protein combination [12,62] and whole-cell [22] vaccines, has been dependent on formulation with strong mucosal adjuvants, such as cholera toxin, which are unlikely to be approved for human use. In contrast, the γ -PN vaccine is non-adjuvanted and still elicits significant protection. We propose that γ -irradiation of pneumococcal whole cells is associated with reduced damage to antigenic epitopes which may facilitate the ability of the γ -irradiated whole-cell-based vaccine to be taken up and processed efficiently by antigen presenting cells, leading to the induction of highly effective immune responses. Furthermore, intranasal administration has limited impact on nasal carriage, highlighting its potential application as a vaccine candidate for human use. In addition, intranasal immunization is highly advantageous due to the fact that it is relatively safe, painless, needle free and a more acceptable vaccination approach to the public. It is also easily deliverable and decreases the risk of disease transmission that can occur through the use of syringes [63].

In summary, the present study describes the development of a non-adjuvanted γ -irradiated whole-cell pneumococcal vaccine that elicits serotype-independent protection against both pneumococcal sepsis and focal pneumonia. We show that the efficacy of our γ -PN vaccine is dependent on both B-cell and IL-17 responses. In particular, we are the first, to our knowledge, to demonstrate the induction of vaccine-induced innate-derived

IL-17-responses. The induction of $\gamma\delta$ T17 cells by γ -PN vaccination may provide a strategic immunization approach to protect, not only against single pneumococcal challenge but also against super-infection by other pathogens.

CLINICAL PERSPECTIVE

- *S. pneumoniae* (the pneumococcus) is the leading cause of morbidity and mortality worldwide. Currently available vaccines provide protection against a limited number of serotypes. The aim of the study was to generate a pneumococcal vaccine with the ability to provide broader 'universal' immunity against a much wider range of serotypes.
- The results of the present study show that intranasal administration of a non-adjuvanted γ -irradiated *S. pneumoniae* whole-cell vaccine provides serotype-independent protection against lethal challenge with *S. pneumoniae* serotypes and this protection is mediated by B-cells and innate IL-17 responses.
- This finding heralds the prospect of being able to provide universal protection against a diversity of *S. pneumoniae*-associated diseases.

AUTHOR CONTRIBUTION

Rachelle Babb designed, performed and analysed experiments, and wrote the manuscript. Austen Chen and Ervin Kara assisted in experimental design, performed experiments and edited the manuscript. Shaun McColl designed some experiments and edited the manuscript. Timothy Hirst, Abiodun Ogunniyi, James Paton and Mohammed Alsharifi supervised the study, designed experiments and wrote the manuscript.

CONFLICT OF INTEREST

Mohammed Alsharifi is the head of vaccine research group at the University of Adelaide and the Chief Scientific Officer of Gamma Vaccine Pty Ltd. Timothy R. Hirst is the Executive chairman of Gamma Vaccine Pty Ltd and an Honorary Professor at the University of Adelaide. This does not alter our adherence to policies on sharing data and materials.

FUNDING

This work was supported by the Australian Research Council (ARC) linkage with Gamma Vaccines Pty Ltd as the Partner Organization [grant number LP120200244 (to J.C.P., A.D.O., M.A. and T.R.H.)]; the Australian postgraduate award [PhD scholarship awarded to R.B.]; and the National Health and Medical Research Council Senior Principal Research Fellow [fellowship awarded to J.C.P.].

REFERENCES

- Kadioglu, A., Weiser, J.N., Paton, J.C. and Andrew, P.W. (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat. Rev. Microbiol.* **6**, 288–301 [CrossRef PubMed](#)
- Obaro, S.K. and Madhi, S.A. (2006) Bacterial pneumonia vaccines and childhood pneumonia: are we winning, refining, or redefining? *Lancet Infect. Dis.* **6**, 150–161 [CrossRef PubMed](#)
- van der Poll, T and Opal, S.M. (2009) Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* **374**, 1543–1556 [CrossRef PubMed](#)
- Robbins, J.B., Austrian, R., Lee, C.J., Rastogi, S.C., Schiffman, G., Henrichsen, J., Makela, P.H., Broome, C.V., Facklam, R.R., Tiesjema, R.H. et al. (1983) Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *J. Infect. Dis.* **148**, 1136–1159 [CrossRef PubMed](#)
- Douglas, R.M., Paton, J.C., Duncan, S.J. and Hansman, D.J. (1983) Antibody response to pneumococcal vaccination in children younger than five years of age. *J. Infect. Dis.* **148**, 131–137 [CrossRef PubMed](#)
- Peeters, C.C., Tenbergen-Meekes, A.M., Haagmans, B., Evenberg, D., Poolman, J.T., Zegers, B.J. and Rijkers, G.T. (1991) Pneumococcal conjugate vaccines. *Immunol. Lett.* **30**, 267–274 [CrossRef PubMed](#)
- Weinberger, D.M., Malley, R. and Lipsitch, M. (2011) Serotype replacement in disease after pneumococcal vaccination. *Lancet* **378**, 1962–1973 [CrossRef PubMed](#)
- Malley, R. and Anderson, P.W. (2012) Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 3623–3627 [CrossRef PubMed](#)
- Moffitt, K.L., Yadav, P., Weinberger, D.M., Anderson, P.W. and Malley, R. (2012) Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. *Vaccine* **30**, 4316–4322 [CrossRef PubMed](#)
- Moffitt, K.L., Gierahn, T.M., Lu, Y.J., Gouveia, P., Alderson, M., Flechtner, J.B., Higgins, D.E. and Malley, R. (2011) T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. *Cell Host Microbe* **9**, 158–165 [CrossRef PubMed](#)
- Liu, Y., Wang, H., Zhang, S., Zeng, L., Xu, X., Wu, K., Wang, W., Yin, N., Song, Z., Zhang, X. and Yin, Y. (2014) Mucosal immunization with recombinant fusion protein DnaJ-DeltaA146Ply enhances cross-protective immunity against *Streptococcus pneumoniae* infection in mice via interleukin 17A. *Infect. Immun.* **82**, 1666–1675 [CrossRef PubMed](#)
- Basset, A., Thompson, C.M., Hollingshead, S.K., Briles, D.E., Ades, E.W., Lipsitch, M. and Malley, R. (2007) Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infect. Immun.* **75**, 5460–5464 [CrossRef PubMed](#)
- Ingram, L.O. (1990) Ethanol tolerance in bacteria. *Crit. Rev. Biotechnol.* **9**, 305–319 [CrossRef PubMed](#)
- Datta, S.K., Okamoto, S., Hayashi, T., Shin, S.S., Mihajlov, I., Fermin, A., Guiney, D.G., Fierer, J. and Raz, E. (2006) Vaccination with irradiated *Listeria* induces protective T cell immunity. *Immunity* **25**, 143–152 [CrossRef PubMed](#)
- Alsharifi, M. and Mullbacher, A. (2010) The gamma-irradiated influenza vaccine and the prospect of producing safe vaccines in general. *Immunol. Cell Biol.* **88**, 103–104 [CrossRef PubMed](#)
- Sommers, C.H. and Rajkowski, K.T. (2011) Radiation inactivation of foodborne pathogens on frozen seafood products. *J. Food Prot.* **74**, 641–644 [CrossRef PubMed](#)
- Marciniak, B., Stawny, M., Kozak, M. and Naskrent, M. (2008) The influence of radiation sterilization on thiamphenicol. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **69**, 865–870 [CrossRef PubMed](#)
- Elliott, L.H., McCormick, J.B. and Johnson, K.M. (1982) Inactivation of Lassa, Marburg, and Ebola viruses by gamma irradiation. *J. Clin. Microbiol.* **16**, 704–708 [PubMed](#)
- Lowy, R.J., Vavrina, G.A. and LaBarre, D.D. (2001) Comparison of gamma and neutron radiation inactivation of influenza A virus. *Antiviral. Res.* **52**, 261–273 [CrossRef PubMed](#)

- 20 Sanakkayala, N., Sokolovska, A., Gulani, J., Hogenesch, H., Sriranganathan, N., Boyle, S.M., Schurig, G.G. and Vemulapalli, R. (2005) Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. *Clin. Diagn. Lab. Immunol.* **12**, 1429–1436 [PubMed](#)
- 21 Chen, A., Mann, B., Gao, G., Heath, R., King, J., Maissoneuve, J., Alderson, M., Tate, A., Hollingshead, S.K., Tweten, R.K. et al. (2015) Multivalent pneumococcal protein vaccines comprising pneumolysin with epitopes/fragments of CbpA and/or PspA elicit strong and broad protection. *Clin. Vaccine Immunol.* **22**, 1079–1089 [CrossRef PubMed](#)
- 22 Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D. and Anderson, P. (2001) Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infect. Immun.* **69**, 4870–4873 [CrossRef PubMed](#)
- 23 Harvey, R.M., Ogunniyi, A.D., Chen, A.Y. and Paton, J.C. (2011) Pneumolysin with low hemolytic activity confers an early growth advantage to *Streptococcus pneumoniae* in the blood. *Infect. Immun.* **79**, 4122–4130 [CrossRef PubMed](#)
- 24 Horton, R.M., Ho, S.N., Pullen, J.K., Hunt, H.D., Cai, Z. and Pease, L.R. (1993) Gene splicing by overlap extension. *Methods Enzymol.* **217**, 270–279 [CrossRef PubMed](#)
- 25 Berry, A.M., Ogunniyi, A.D., Miller, D.C. and Paton, J.C. (1999) Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infect. Immun.* **67**, 981–985 [PubMed](#)
- 26 McAllister, L.J., Tseng, H.J., Ogunniyi, A.D., Jennings, M.P., McEwan, A.G. and Paton, J.C. (2004) Molecular analysis of the *psa* permease complex of *Streptococcus pneumoniae*. *Mol. Microbiol.* **53**, 889–901 [CrossRef PubMed](#)
- 27 Paton, J.C., Lock, R.A. and Hansman, D.J. (1983) Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect. Immun.* **40**, 548–552 [PubMed](#)
- 28 Trappetti, C., van der Maten, E., Amin, Z., Potter, A.J., Chen, A.Y., van Mourik, P.M., Lawrence, A.J., Paton, A.W. and Paton, J.C. (2013) Site of isolation determines biofilm formation and virulence phenotypes of *Streptococcus pneumoniae* serotype 3 clinical isolates. *Infect. Immun.* **81**, 505–513 [CrossRef PubMed](#)
- 29 Montgomery, C.P., Daniels, M., Zhao, F., Alegre, M.L., Chong, A.S. and Daum, R.S. (2014) Protective immunity against recurrent *Staphylococcus aureus* skin infection requires antibody and interleukin-17A. *Infect. Immun.* **82**, 2125–2134 [CrossRef PubMed](#)
- 30 Lu, Y.J., Gross, J., Bogaert, D., Finn, A., Bagrade, L., Zhang, Q., Kolls, J.K., Srivastava, A., Lundgren, A., Forte, S. et al. (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog* **4**, e1000159 [CrossRef PubMed](#)
- 31 Haylock-Jacobs, S., Comerford, I., Bunting, M., Kara, E., Townley, S., Klingler-Hoffmann, M., Vanhaesebroeck, B., Puri, K.D. and McColl, S.R. (2011) PI3Kdelta drives the pathogenesis of experimental autoimmune encephalomyelitis by inhibiting effector T cell apoptosis and promoting Th17 differentiation. *J. Autoimmun.* **36**, 278–287 [CrossRef PubMed](#)
- 32 Brundage, J.F. (2006) Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. *Lancet Infect. Dis.* **6**, 303–312 [CrossRef PubMed](#)
- 33 Wu, T., Hu, Y., Lee, Y.T., Bouchard, K.R., Benechet, A., Khanna, K. and Cauley, L.S. (2014) Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J. Leukoc. Biol.* **95**, 215–224 [CrossRef PubMed](#)
- 34 Bosch, A.A., Biesbroek, G., Trzcinski, K., Sanders, E.A. and Bogaert, D. (2013) Viral and bacterial interactions in the upper respiratory tract. *PLoS Pathog* **9**, e1003057 [CrossRef PubMed](#)
- 35 McDaniel, L.S., Sheffield, J.S., Delucchi, P. and Briles, D.E. (1991) PspA, a surface protein of *Streptococcus pneumoniae*, is capable of eliciting protection against pneumococci of more than one capsular type. *Infect. Immun.* **59**, 222–228 [PubMed](#)
- 36 Briles, D.E., Tart, R.C., Wu, H.Y., Ralph, B.A., Russell, M.W. and McDaniel, L.S. (1996) Systemic and mucosal protective immunity to pneumococcal surface protein, A. *Ann. N. Y. Acad. Sci.* **797**, 118–126 [CrossRef PubMed](#)
- 37 Sun, K., Salmon, S.L., Lotz, S.A. and Metzger, D.W. (2007) Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. *Infect. Immun.* **75**, 1196–1202 [CrossRef PubMed](#)
- 38 Yamada, M., Gomez, J.C., Chugh, P.E., Lowell, C.A., Dinauer, M.C., Dittmer, D.P. and Doerschuk, C.M. (2011) Interferon-gamma production by neutrophils during bacterial pneumonia in mice. *Am. J. Respir. Crit. Care Med.* **183**, 1391–1401 [CrossRef PubMed](#)
- 39 Richards, L., Ferreira, D.M., Miyaji, E.N., Andrew, P.W. and Kadioglu, A. (2010) The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology* **215**, 251–263 [CrossRef PubMed](#)
- 40 Zhang, Z., Clarke, T.B. and Weiser, J.N. (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J. Clin. Invest.* **119**, 1899–1909 [PubMed](#)
- 41 Shin, H. and Iwasaki, A. (2013) Tissue-resident memory T cells. *Immunol. Rev.* **255**, 165–181 [CrossRef PubMed](#)
- 42 Hogan, R.J., Zhong, W., Usherwood, E.J., Cookenham, T., Roberts, A.D. and Woodland, D.L. (2001) Protection from respiratory virus infections can be mediated by antigen-specific CD4(+) T cells that persist in the lungs. *J. Exp. Med.* **193**, 981–986 [CrossRef PubMed](#)
- 43 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. et al. (2010) Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. *PLoS Pathog.* **6**, e1001191 [CrossRef PubMed](#)
- 44 Michel, M.L., Keller, A.C., Paget, C., Fujio, M., Trottein, F., Savage, P.B., Wong, C.H., Schneider, E., Dy, M. and Leite-de-Moraes, M.C. (2007) Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* **204**, 995–1001 [CrossRef PubMed](#)
- 45 Nakasone, C., Yamamoto, N., Nakamatsu, M., Kinjo, T., Miyagi, K., Uezu, K., Nakamura, K., Higa, F., Ishikawa, H., O'Brien, R. L. et al. (2007) Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. *Microbes Infect.* **9**, 251–258 [CrossRef PubMed](#)
- 46 Eisenberg, Jr, G.H. and Osterman, J.V. (1979) Gamma-irradiated scrub typhus immunogens: broad-spectrum immunity with combinations of rickettsial strains. *Infect. Immun.* **26**, 131–136 [PubMed](#)
- 47 Weiser, J.N., Austrian, R., Sreenivasan, P.K. and Masure, H.R. (1994) Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect. Immun.* **62**, 2582–2589 [PubMed](#)
- 48 Weiser, J.N., Markiewicz, Z., Tuomanen, E.I. and Wani, J.H. (1996) Relationship between phase variation in colony morphology, intrastain variation in cell wall physiology, and nasopharyngeal colonization by *Streptococcus pneumoniae*. *Infect. Immun.* **64**, 2240–2245 [PubMed](#)
- 49 Furuya, Y., Regner, M., Lobigs, M., Koskinen, A., Mullbacher, A. and Alsharifi, M. (2010) Effect of inactivation method on the cross-protective immunity induced by whole 'killed' influenza A viruses and commercial vaccine preparations. *J. Gen. Virol.* **91**, 1450–1460 [CrossRef PubMed](#)
- 50 McCool, T.L. and Weiser, J.N. (2004) Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. *Infect. Immun.* **72**, 5807–5813 [CrossRef PubMed](#)

- 51 Lipsitch, M., Whitney, C.G., Zell, E., Kaijalainen, T., Dagan, R. and Malley, R. (2005) Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? *PLoS Med.* **2**, e15 [CrossRef PubMed](#)
- 52 Malley, R., Trzcinski, K., Srivastava, A., Thompson, C.M., Anderson, P.W. and Lipsitch, M. (2005) CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 4848–4853 [CrossRef PubMed](#)
- 53 Cao, J., Wang, D., Xu, F., Gong, Y., Wang, H., Song, Z., Li, D., Zhang, H., Zhang, L., Xia, Y. et al. (2014) Activation of IL-27 signalling promotes development of postinfluenza pneumococcal pneumonia. *EMBO Mol. Med.* **6**, 120–140 [CrossRef PubMed](#)
- 54 Robinson, K.M., Kolls, J.K. and Alcorn, J.F. (2015) The immunology of influenza virus-associated bacterial pneumonia. *Curr. Opin. Immunol.* **34**, 59–67 [CrossRef PubMed](#)
- 55 Nichol, K.L. (1999) The additive benefits of influenza and pneumococcal vaccinations during influenza seasons among elderly persons with chronic lung disease. *Vaccine* **17** (Suppl 1), S91–S93 [CrossRef PubMed](#)
- 56 Kudva, A., Scheller, E.V., Robinson, K.M., Crowe, C.R., Choi, S.M., Slight, S.R., Khader, S.A., Dubin, P.J., Enelow, R.I., Kolls, J.K. and Alcorn, J.F. (2011) Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J. Immunol.* **186**, 1666–1674 [CrossRef PubMed](#)
- 57 Kadioglu, A., Cuppone, A.M., Trappetti, C., List, T., Spreafico, A., Pozzi, G., Andrew, P.W. and Oggioni, M.R. (2011) Sex-based differences in susceptibility to respiratory and systemic pneumococcal disease in mice. *J. Infect. Dis.* **204**, 1971–1979 [CrossRef PubMed](#)
- 58 Kao, C.Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R.W. and Wu, R. (2004) IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *J. Immunol.* **173**, 3482–3491 [CrossRef PubMed](#)
- 59 Lee, H.Y., Andalibi, A., Webster, P., Moon, S.K., Teufert, K., Kang, S.H., Li, J.D., Nagura, M., Ganz, T. and Lim, D.J. (2004) Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*. *BMC Infect. Dis.* **4**, 12 [CrossRef PubMed](#)
- 60 Li, W., Moltedo, B. and Moran, T.M. (2012) Type I interferon induction during influenza virus infection increases susceptibility to secondary *Streptococcus pneumoniae* infection by negative regulation of gammadelta T cells. *J. Virol.* **86**, 12304–12312 [CrossRef PubMed](#)
- 61 van Gils, E.J., Hak, E., Veenhoven, R.H., Rodenburg, G.D., Bogaert, D., Bruin, J.P., van Alphen, L. and Sanders, E.A. (2011) Effect of seven-valent pneumococcal conjugate vaccine on *Staphylococcus aureus* colonisation in a randomised controlled trial. *PLoS One* **6**, e20229 [CrossRef PubMed](#)
- 62 Ogunniyi, A.D., Grabowicz, M., Briles, D.E., Cook, J. and 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 [CrossRef PubMed](#)
- 63 Riese, P., Sakthivel, P., Trittel, S. and Guzman, C.A. (2014) Intranasal formulations: promising strategy to deliver vaccines. *Expert Opin. Drug Deliv.* **11**, 1619–1634 [CrossRef PubMed](#)

Received 1 October 2015/25 January 2016; accepted 1 February 2016

Accepted Manuscript online 1 February 2016, doi: 10.1042/CS20150699