Development of a Novel Co-vaccination Approach for Pneumococcal and Influenza Infections

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ABBREVIATIONS

A_{405}	Absorbance at 405 nm
A_{450}	Absorbance at 450 nm
ABC	ATP-binding cassette
ANT3	Adenine nucleotide translocator 3
APC	Antigen presenting cells
A/PC	A/Port Chalmers/1/73 [H3N2]
A/PR8	A/Puerto Rico/8/34 [H1N1] influenza strain
CbpA	Choline-binding protein A
CD	Cluster of differentiation
CFU	Colony forming unit
ChoP	Phosphorylcholine
CPG ODN	Cytosine phosphate guanosine oligodeoxynucleotides
CPS	Capsular polysaccharides
CRP	C-reactive protein
CTL	Cytotoxic T lymphocytes
CT	Cholera toxin
DC	Dendritic cell
DI	Dry ice
DMEM	Dulbecco' Modified Eagle's Medium
DTaP	Diphtheria-tetanus-acellular pertussis vaccine
Eno	Enolase
FACS	Fluorescent activated cell sorting
FCS	Foetal Calf Serum
FcR	Fc Receptor
FFI	Focus forming inhibition
Foxp3	Forkhead box P3
HA	Hemagglutinin
Hep B	Hepatitis B virus
Hib	*Haemophilus influenza* type b
HPV	Human papilloma virus
HRP	Horse Radish Peroxidase
IFN-I	Type I Interferon (α/β)
IL-	Interleukin
IFN	Interferon
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IN	Intranasally
IP	Intraperitoneally
IPD	Invasive pneumococcal disease
IPV	Inactivated poliovirus
IRF	Interferon regulatory factors
IV	Intravenously
KO	Knock out
kGy	kiloGray
LAIV	Live attenuated influenza vaccines
LT	Labile toxin
LytA	Autolysin

M1/2	Matrix protein 1/2
MARCO	Macrophage receptor with collagenous structure
M cells	Microfold cells
MC	Mannosylated Chitosan
MFI	Mean fluorescence intensity
mg	milligram/s
MHC	Major histocompatibility complex
mL	millilitre/s
MMR	Measles, Mumps and Rubella vaccine
MPL	Monophosphoryl lipid A
NA	Neuraminidase
NALT	Nasopharynx-associated lymphoid tissue
NEP	Nuclear Export Protein
NF-	Nuclear Factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cell
NKT	Natural Killer T cell
NLR	Nod-like receptor/s
NP	Nucleoprotein
NPP	Nucleoprotein peptide
NS1	Non-structural protein 1
OD	Optical density
PA	Acidic polymerase
PAFr	Platelet-activating factor receptor
PAMPs	Pathogen associated molecular patterns
PavA	Pneumococcal adhesion and virulence A
PB1/2	Basic polymerase protein 1/2
PBS	Phosphate buffered saline
PBPs	Penicillin Binding proteins
PCR	Polymerase chain reaction
PCVs	Pneumococcal conjugate vaccines
PdT	Pneumolysin mutant
PhtD	Pneumococcal histidine triad D
PhtE	Pneumococcal histidine triad E
Ply	Pneumolysin
PKR	Protein Kinase R
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
PRR	Pattern recognition receptor
RT	Room temperature
RIG	Retinoic acid–inducible gene like receptors
S. pneumoniae	*Streptococcus pneumoniae*
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SFV	Semliki Forest Virus
ssRNA	Single-stranded ribonucleic acid

$TCID_{50}$	50% tissue culture infective dose
TCR	T Cell receptor
Tfh	Follicular $CD4^+$ T helper
Th17	$CD4^+$ T helper 17
Th1	$CD4^+$ T helper 1
THY	Todd-Hewitt broth
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TRM	Tissue resident memory
WC	Whole-cell
WCV	Whole-cell vaccine
WT	Wild type
α-GalCer	Alpha-galactosylceramide
μg	microgram/s
μL	microlitre/s
γδ T	Gamma-delta T
γδ T17	Gamma-delta T cells secreting $IL\text{-}17^+$
γ-FLU	Gamma-irradiated influenza vaccine
γ-PN	Gamma-irradiated *Streptococcus pneumoniae* vaccine
γ-SFV	Gamma-irradiated Semliki Forest vaccine

DECLARATION

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Rachelle Babb

Date

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PATENTS, PUBLICATIONS AND CONFERENCE PRESENTATIONS ARISING FROM THIS THESIS

PATENTS		
Patent application No	**Title**	**Inventors**
PCT/AU2016/050231	Streptococcal Vaccine	Rachelle Babb, Mohammed Alsharifi, Austen Yannis Chen Shannon Christa David, Timothy Raymond Hirst, Abiodun David Ogunniyi, James Cleland Paton

PUBLICATIONS		
Thesis chapter	**Title**	**Publication status**
3	Intranasal vaccination with gamma-irradiated Streptococcus pneumoniae whole-cell vaccine provides serotype-independent protection mediated by B cells and innate IL-17 responses *Appendix	Published
5	Enhanced protective CD4+ T cell responses to a serotype independent pneumococcal vaccine when combined with an inactivated influenza vaccine	In submission

CONFERENCE PRESENTATIONS	
• The 12th European Meeting on the Molecular Biology of the Pneumococcus (Oxford University, Oxford, UK, 2015).	Poster
• School of Biological Sciences Research Symposium (University of Adelaide, Adelaide, Australia, 2013-2014).	Presentation

ABSTRACT

Streptococcus pneumoniae and influenza are the world's foremost bacterial and viral respiratory pathogens. In addition to their individual clinical significance, co-infection with these pathogens enhances disease progression and is associated with substantially increased mortality rates. Vaccination is the best preventative method to control disease caused by individual pathogens as well as co-infection. Gamma-irradiation is considered a safe sterilization method, used routinely to sterilize medical devices, pharmaceuticals and most commonly food products. It can also be utilised as an inactivation technique to generate whole cell bacterial and viral vaccines with minimal impact on pathogen structure and antigenic determinants. This study presents the first evidence illustrating the use of this inactivation technique for development of a mucosal *S. pneumoniae* whole cell vaccine (γ-PN). Gamma-irradiation was utilised to inactivate an unencapsulated *S. pneumoniae* strain Rx1 with an unmarked deletion of the autolysin gene and with the pneumolysin gene replaced with an allele encoding a non-toxic pneumolysoid. Intranasal administration of mice with γ-PN without an adjuvant was shown to elicit serotype-independent protection against pneumococcal challenge in models of sepsis and pneumonia. In particular, vaccine efficacy was shown to be reliant on B cells and IL-17 responses. Importantly, immunisation promoted IL-17 production by γδ T cells, as opposed to conventional Th17 cells commonly reported with other pneumococcal whole cell vaccines. Moreover, this study also illustrated that the immunogenicity and protective efficacy of the γ-PN vaccine can be enhanced in the presence of the mucosal adjuvant, cholera toxin.

In addition, this study describes a novel combination vaccine approach comprising inactivated whole bacterial cells and whole virions to *S. pneumoniae* and influenza respectively. In this study mice were co-immunised intranasally with the un-adjuvanted γ-PN vaccine and a gamma-irradiated influenza vaccine (γ-FLU). Interestingly, co-immunisation was shown to enhance γ-PN vaccine efficacy and immunogenicity against virulent pneumococcal challenge, which was dependent on $CD4^+$ T cell responses. In contrast to vaccination with γ-PN alone, co-immunisation enhanced pneumococcal-specific effector Th17 and Th1 memory cells, promoted development of $CD4^+$ tissue-resident memory cells, and enhanced pneumococcus-specific antibody responses. In addition, this combination approach was shown to elicit significant protection against lethal influenza challenge, as well

as against co-infection with both influenza and *S. pneumoniae.* These data support the notion that γ-FLU exhibits adjuvant-like properties to enhance immunogenicity of a co-administered vaccine without compromising pathogen-specific immune responses. Future work will be focused on clinical development of individual and combination vaccines.

CHAPTER 1

Introduction

Streptococcus pneumoniae (the pneumococcus) is one of the most challenging upper respiratory bacterial pathogens in humans. In addition to causing a significant global disease burden from individual infections, co-infection with other respiratory pathogens such as influenza is associated with higher morbidity and mortality rates. Therefore, vaccination represents an important prevention measure. This review will outline the pathogenesis of *S. pneumoniae* and influenza infection, the underlying causes of co-infection and current vaccination approaches to each of these respiratory pathogens. This review will also introduce the concept of gamma-irradiation as a promising inactivation technique for vaccine development.

1.1. *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive bacterium, which is known to be the leading cause of otitis media, pneumonia, bacteraemia and meningitis worldwide (1). It is a common component of the nasopharyngeal microflora and is estimated to be carried asymptomatically by approximately 60% of the population, with higher carriage rates among children under 6 years old (2). Serotype classification of *S. pneumoniae* is defined by the capsular polysaccharides (CPS) that constitute the anti-phagocytic capsule forming the outermost layer of the bacterium (3, 4). 93 structurally distinct CPS have been recognized to date, and these may differ in their ability to cause disease (5).

1.1.1 Significance

Pneumococcal disease is highly prevalent in young children under the age of 2, the elderly (>65 yrs) and immuno-compromised individuals with underlying illnesses such as chronic heart disease, lung disease and asplenia (6-8). In addition, invasive pneumococcal disease has also been reported to be 25-50 fold higher in patients infected with human immunodeficiency virus (HIV) (9-11). Furthermore, co-infection with other pathogens such as influenza virus can also trigger progression of asymptomatic pneumococcal colonization into invasive disease, which is commonly observed during influenza pandemics (12). In 2006, the World Health Organization (WHO) estimated that approximately 1.5 million people die annually as a result of pneumococcal disease, with 1 million of these deaths occurring in children under the age of 5 in developing countries (1, 13-15). Indeed, it has been reported that for every 100,000 people in developing countries, more than 25-30 cases of invasive pneumococcal

disease (bacteraemia and meningitis) occur in people of all ages, with more than 50 cases occurring in the elderly (>65 yrs old) (16). Global estimates of pneumococcal disease burden in the year 2000 reported 14.5 million cases of pneumococcal infection, with countries within Asia and Africa accounting for 66% of all cases. Specifically, 96% of these cases were reported to be pneumonia (14). Therefore, these studies illustrate the high prevalence and severity of pneumococcal disease in developing countries.

1.1.2 Pneumococcal carriage and disease

Humans are the only known natural reservoir for harbouring *S. pneumoniae.* Early in life, *S. pneumoniae* colonizes the nasopharynx of infants within a few months of birth. Depending on the immunogenicity of particular serotypes, certain strains can colonize the nasopharynx for approximately a month or can last up to a year. Eventually as immunity develops towards colonizing pneumococci, these serotypes are replaced with alternative strains and this process continues until adulthood (17, 18). It has been reported that the majority of children (~95%) can be colonized with up to 6 different serotypes by the age of 2 (19). Interestingly, it has also been shown that colonizing serotypes differ significantly between children (less than 5 yrs) and adults (20). Furthermore, the degree of encapsulation has been shown to influence rates of carriage, with encapsulated pneumococci associated with higher carriage rates in comparison to significantly low carriage rates for unencapsulated strains (21).

Pneumococcal disease can be divided into non-invasive and invasive disease. Non-invasive disease is associated with localised infection within particular niches, such as infection of the sinuses (sinusitis), the inner ear (otitis media) and the lungs (pneumonia). Of all of the non-invasive diseases, pneumonia poses the highest risk of morbidity and mortality. In order to establish pneumonia, pneumococci migrate from the nasopharynx by aspiration into the alveoli and establish infection, causing excessive inflammation. This results in symptoms such as fever, cough and tachypnoea. In healthy individuals, pneumococcal pneumonia is readily treatable, leading to complete recovery. However, in individuals with complicated health issues, such as those with lung disease or infected with other respiratory infections, pneumococcal pneumonia can often result in death. Alternatively, *S. pneumoniae* can progress to invasive disease, whereby the bacteria can migrate into niches such as brain and blood to cause meningitis and bacteraemia respectively. Bacteraemia can predispose pneumococci to cross the blood-brain barrier and cause meningitis, which is an inflammation

of the meninges that cover the brain and spinal cord. Meningitis initially results in symptoms such as fever, irritability, vomiting and an altered mental state. Progression of the disease leads to seizures, focal neuropathology and coma (22). Epidemiological studies have found that pneumococci that express a large capsule are highly virulent and more likely to progress to invasive disease. In contrast, strains that are unencapsulated are generally avirulent (22-24), although some of these strains have been reported to be associated with superficial infections such as conjunctivitis (25, 26). A study evaluating the relationship between serotypes and mortality rates found that serotypes 1, 7F and 8 were associated with lower mortality rates in comparison to that observed with serotype 3, 6A, 6B, 9N and 19F (27). Nevertheless, all non-invasive and invasive pneumococcal disease results in a high medical and economical burden.

1.1.3 Antibiotic treatment

Since the 1940s, penicillin was the traditional antibiotic used for treatment of *S. pneumoniae* infections. Prior to the use of penicillin, approximately 20%, 50% and between 80-100% mortality rates were associated with pneumococcal pneumonia, bacteraemia and meningitis, respectively (28). However after introduction of penicillin, these mortality rates significantly reduced to 5%, 20% and 30%, respectively (29). With continued overuse of antibiotics throughout the years, pneumococcal strains began to evolve mechanisms to resist the actions of antibiotics. The first penicillin-resistant pneumococcal strain was isolated in 1967 in Papua New Guinea followed by reports from South Africa, Israel, Poland, Spain and the US (30) and by 1974, penicillin-resistant clinical isolates were detectable worldwide (31). Pneumococci became multi-resistant against not only penicillin but also other antibiotics such as erythromycin, clindamycin, tetracycline, streptomycin and chloramphenicol (32, 33). Therefore, it is important that antibiotic resistance patterns are carefully monitored and that on-going research to develop new antimicrobials effective against *S. pneumoniae* continues. Nevertheless, the rapid evolution and dissemination of resistant pneumococci underscore the need for more effective pneumococcal vaccines, as discussed later.

1.1.4 Pathogenesis

Transmission of the bacterium generally occurs via aerosol droplets or direct contact with infectious secretions. Upon entrance through the nasal cavity, components of the cell wall

such as phosphorylcholine (ChoP) mediate direct attachment to the respiratory epithelium via binding to the platelet-activating factor receptor (PAFr) (34). In addition, hyaluronidase secreted by *S. pneumoniae* degrades hyaluronic acid, which is an important component of host connective tissue and the extracellular matrix. This assists in the binding of bacterial surface proteins such as pneumococcal adhesion and virulence A (PavA) and enolase (Eno) to host's fibronectin and plasminogen, respectively (35-37), which further facilitates adherence to the epithelium. The enzyme neuraminidase also plays a critical role as it cleaves N-acetylneuraminic acid to decrease the viscosity of mucus lining the upper respiratory tract and exposes N-acetyl-glycosamine receptors on host epithelial cells through cleaving terminal sialic acid from glycolipids, glycoproteins, and oligosaccharides (38). Furthermore, pneumococcal surface protein C (PspC), also known as choline-binding protein A (CbpA), promotes translocation of the bacterium across the epithelium via interactions with human secretory component located on the polymeric immunoglobulin receptor (39). Overall, synergistic activities of these virulence factors promote successful nasopharyngeal colonization (Figure 1.1).

In addition to invading the respiratory epithelium, *S. pneumoniae* utilises many virulence factors to evade the hosts' immune defence. For example, the capsule, which is the most potent virulence factor, forms an inert shield that strongly impedes opsonisation by preventing the binding of IgG and complement component deposition of C3b/iC3b on the bacterial surface by both the classical and alternative complement pathways. As a result, it inhibits the process of phagocytosis mediated by both complement and Fcγ receptors on phagocytic cells (40). In addition, the capsule has also been reported to reduce trapping of the bacteria by neutrophil extracellular traps (41). Another crucial virulence factor is the toxin pneumolysin (Ply), which is a pore forming toxin released from the bacterium through the action of the enzyme autolysin (LytA) (42). Ply contributes greatly to the pathogenesis of *S. pneumoniae* via many mechanisms; it can bind to cholesterol within the membranes of target cells leading to the formation of large pores and cytolysis (43), it has been shown to inhibit the ciliary beating of respiratory epithelium (44), it is involved with activation of the complement pathway (45) and it has been shown to reduce lymphocyte proliferation (46, 47). Ply has also been shown to promote the production of inflammatory mediators such as, TNF-α, IL-6, as well as the chemokines CXCL1 and CXCL2. This combination of pro-inflammatory cytokines and chemokines facilitates the induction of an inflammatory response and contributes to the influx of neutrophils during early stages of infection (48, 49).

In addition, pneumococcal surface protein A (PspA) located on the cell wall is also pivotal for virulence. It has a high negative charge, which is important for interfering with complement component C3b deposition on the bacterial surface (50). It has also been reported to bind to lactoferrin, which is an iron storage glycoprotein, to increase the amount of iron available for maintaining growth of the bacterium (51, 52). Similarly, aside from its major role during bacterial invasion, CbpA also interferes with the complement system by recruiting negative regulators serum factor H and C4-binding protein to prevent activation of the alternative and classical pathways, respectively (53, 54). Overall, a combination of these virulence factors and many others not listed here are essential for the persistence of pneumococci within the host.

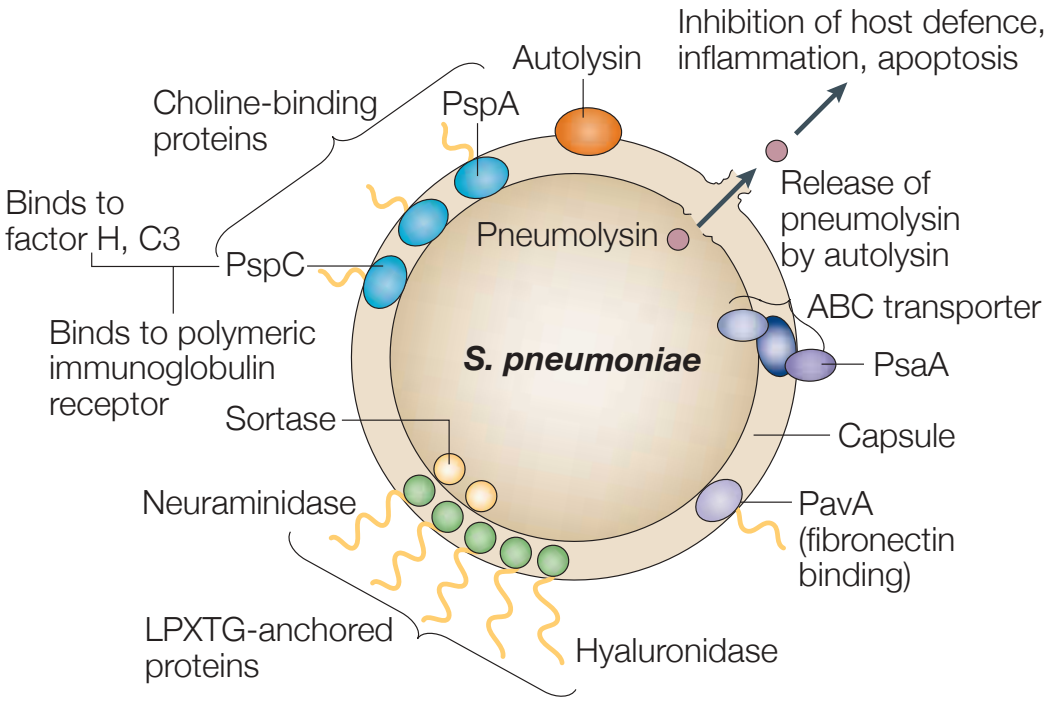


Figure 1.1: *S. pneumoniae* structure and virulence factors (55).

1.1.5 Immunity

1.1.5.1 Innate immunity

Upon infection, *S. pneumoniae* is recognised by an array of germline-encoded pattern recognition receptors (PRRs). PRRs such as Toll-like receptors (TLRs) are capable of detecting an extensive range of conserved microbial signatures termed pathogen associated molecular patterns (PAMPs). Recognition of PAMPs by TLRs trigger signalling pathways

that result in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Interferon regulatory factors (IRF) 3/7, which lead to the synthesis of proinflammatory chemokines/cytokines such as interleukin (IL-) 8, tumor necrosis factor (TNF-α) and interferon type I (IFN-I) (56). During pneumococcal infection, components of the cell wall such as lipoteichoic acid and lipoproteins are recognized by TLR2 (57), bacterial DNA containing unmethylated CpG motifs are recognized by TLR9 (58) and the toxin pneumolysin has been shown to interact with TLR4 (59). In addition, gene knock out (KO) studies in mice have illustrated the importance of signalling through TLR2, TLR4 and TLR9 in defence against pneumococcal infection. TLR2 signalling has been shown to play a critical role in protection against colonization (60). It has also been demonstrated to be important for clearance of bacteria in pneumococcal meningitis as TLR2 KO mice were associated with increased blood brain barrier permeability, CNS bacterial burden and inflammation (61). TLR4 signalling has also been demonstrated to be important for protective immune responses against pneumococcal pneumonia. Particularly, evaluation of Ply-mediated TLR4 signalling showed that TLR4 deficient mice were unable to control proliferation in the nasopharynx, which caused them to be highly susceptible to invasive disease (59, 62). Moreover, TLR9 has been shown be important in the lower respiratory tract during the early stages of pneumococcal infection. TRL9 was not involved in the recruitment of immune cells, but rather had a pivotal role in the phagocytic clearance of bacteria by resident macrophages (58).

In addition to TLRs, Nod-like receptors (NLR's) also play important roles in the recognition of bacterial components during pneumococcal infection. NLR's are intracellular PRR's within the cytosol which function via activation of the multi-protein complex called the inflammasome, which in turn promotes the cleavage and secretion of cytokines IL-1β and IL-18. These powerful proinflammatory cytokines display pleiotropic functions to defend against bacterial infections (63, 64). During *S. pneumoniae* infection, the toxin Ply readily activates the NLRP3 inflammasome to mediate the production of IL-1β in macrophages and DCs (65). Other NLR's including NOD2 is also important in host defence. It has been shown that upon recognition of bacterial cell wall components that contain muramyl dipeptide, NOD2 signalling induces the production of the chemokine attractant CCL2, which is essential for promoting macrophage recruitment (66). Pyrin and HIN200 domain-containing (PYHIN) proteins, another family of PRRs that also facilitate formation of the inflammasome (67), have been reported to play a role during pneumococcal infection. In particular, the PYHIN protein AIM2, which binds to cytosolic double-stranded DNA, is critical for

activation of caspase 1 in macrophages following infection with *S. pneumoniae,* leading to the secretion of IL-18 and IL-1β (68).

Furthermore, aside from PRR signaling, innate immune cell populations such as NKT cells and γδ T cells are also crucial during pneumococcal infections. In particular, these cells have been shown to stimulate the production of TNF-α and CXCL2 to promote neutrophil-mediated host defense against bacteria (69, 70).

Overall, the response to pneumococcal infection is multifaceted and involves many aspects of the innate immune system, which shapes the adaptive immune response against *S. pneumoniae*.

1.1.5.2 Adaptive immunity

Antibody responses

During *S. pneumoniae* infection, antibodies generated against the CPS are particularly important for mediating bacterial clearance by the process of opsonophagocytosis and have been proven to provide protection in humans and in various animal species (71, 72). In addition, antibodies can also be generated against the subcapsular antigens that lie underneath or are interspersed between the CPS such as PspA, CbpA, pneumococcal surface antigen A (PsaA) and pneumococcal histidine triad protein D (PhtD) and E (PhtE). However, unlike the serotype specific anti-capsular antibodies, antibodies directed against the subcapsular antigens can target all serotypes and have also been demonstrated to be capable of mediating immunity to pneumococcal infections (73-77). Besides the prominent role of antibodies in opsonising pneumococci for phagocytic clearance, there is also evidence to show that the capsular polysaccharide conjugate vaccines (PCV)'s can induce non-opsonic antibodies to the CPS. Studies evaluating the efficacy of non-opsonic antibodies have demonstrated that these antibodies are protective against lethal pneumococcal challenge with serotype 3 and 8 (78, 79). Further work investigating the efficacy of the human non-opsonic antibody, 1E2, showed that in contrast to the opsonic antibody 7A9, 1E2 reduced colonization and prevented bacterial dissemination throughout the host. Interestingly, it was found to mediate protection in an Fc-independent manner. Although, the precise mechanisms of how these non-opsonic antibodies mediate protection *in vivo* is currently under investigation, bacterial agglutination and enhanced fratricide have been proposed (80).

CD4$^+$ T cell immunity

Aside from the process of complement-mediated opsonophagocytosis, CD4$^+$ T helper cell responses have been shown to be another important component in adaptive immunity against *S. pneumoniae*. The subset of CD4$^+$ T cells that produces IL-17, known as T helper 17 (Th17) cells are predominately involved in host defence against extracellular pathogens and fungi (81). In recent years, it has been confirmed that the Th17 signature cytokine IL-17 is essential for protection against *S. pneumoniae* infection, as neutralization of IL-17 was shown to abrogate protection following mucosal immunization with a whole-cell vaccine (82, 83). CD4$^+$ T cells of the IL-17 lineage have been clearly demonstrated to be required for early recruitment of neutrophils and macrophages during the initial stages of colonisation. This process subsequently facilitates the clearance of invading pneumococci, by opsonisation-dependent mechanisms (84, 85). In addition, Moffitt and colleagues used a proteomic approach to identify antigens that stimulated production of IL-17 in CD4$^+$ T cells from mice immunized with the whole-cell vaccine. They demonstrated that there was a hierarchy in antigens promoting IL-17 responses and proposed that these antigens differ to those recognized by protective antibodies. In particular, they found that the antigens SP0148, SP2108 and SP0882 promoted robust Th17 responses and conferred significant protection against colonization following intranasal immunization (86). Further work evaluating SP2108 and SP0148, which are substrate-binding lipoproteins of ATP-binding cassette (ABC) transporter complexes, showed that the lipid moieties of these proteins are critical in mediating TLR2-dependent protection against nasopharyngeal carriage (87).

Taken together, these studies demonstrate the importance of Th17 responses during pneumococcal infection and therefore induction of these cells would be highly desirable for eliciting vaccine-induced immunity to *S. pneumoniae.*

1.1.5.3 Complement and antibody-mediated immunity

The host's major defence mechanism against pneumococcal infections is the induction of antibody-initiated complement-dependent opsonisation (88), whereby phagocytosis and intracellular destruction of pneumococci is highly dependent on the presence of opsonins, i.e. immunoglobulins and complement (89). In general, all classes of the pneumococcal-specific antibodies (IgM, IgG or IgA) can initiate the process of complement-dependent opsonophagocytosis through the binding of C1q indirectly to Fc portions of the antibody-

antigen complexes. This results in activation of the classical pathway of the complement system, which is found to be the most dominant pathway for protection against the pneumococcus (90, 91). In addition to antibodies, other factors can also lead to classical pathway activation during pneumococcal infection. C-reactive protein (CRP) has been shown to bind to ChoP within the bacterial cell wall resulting in an interaction with C1q (92). Furthermore, C1q itself has also been shown to directly bind to the bacterial surface causing activation of the complement pathway (93). Overall, activation of the complement system leads to the deposition of complement components C3b and iC3b on the bacterial cell surface. This triggers a cascade that ultimately causes phagocytic cells to mediate effective clearance of opsonized bacteria by binding to the Fc fragments of IgG/IgM and complement components (C3b and iC3b) via their complementary receptors (2). The alternative pathway can also be activated upon pneumococcal infection, but to a lesser extent (90, 94). As mentioned earlier, pneumococcal virulence factors such as PspA and CbpA are well known for their ability to interfere with complement activation (50, 53).

1.1.6 Pneumococcal vaccines

1.1.6.1 Capsular Polysaccharide vaccines

The CPS are considered the major virulence factor and dominant surface antigen of *S. pneumoniae,* therefore 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 (95). This vaccine is immunogenic in adults and children older than 5 years, but is poorly immunogenic in children less than 5 years old (96). CPS are T-cell independent antigens that are poorly immunogenic and do not induce robust long-term humoral memory. This has been reported to be responsible for the lack of protection in younger children. These limitations led to development of pneumococcal conjugate vaccines (PCV)'s, whereby the CPS is conjugated to a protein carrier to promote induction of T cell-dependent immunity. This lead to the initial development of the 7 valent pneumococcal conjugate vaccine (PCV7) containing serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. The most recent pneumococcal conjugate vaccine (PCV13) (Prevnar 13®) covers 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F & 23F) and has been in routine use since 2010. These preparations elicit strong immunity in infants and

young children (97). However, high cost and technical complexity restricts the number of serotypes that can be included in existing vaccine combinations. In addition, although there has been a significant reduction in the incidence of invasive disease corresponding to particular vaccine-included serotypes, these current vaccine formulations do not protect against non-vaccine serotypes. This has resulted in an increased prevalence of non-vaccine serotypes in pneumococcal carriage and disease, a phenomenon known as 'serotype replacement' (1, 98).

Serotype replacement

Serotype replacement refers to the replacement of vaccine type pneumococci harboured within the nasal microflora with alternative serotypes. Replacement of these particular strains due to protective immunity induced by current vaccines, has consequently increased the incidence of pneumococcal carriage and disease with non-vaccine serotypes (98-100) as well as other colonizing bacteria such as *Staphylococcus aureus (S. aureus)* (101). This mitigates the benefits of vaccination regimes and has affected the overall aim of reducing the incidence of pneumococcal invasive disease. For example, although the introduction of PCV7 in Australia significantly reduced invasive disease in children under the age of 2 by 74% during the period of 2002-2007, the incidence of invasive disease due to non-vaccine serotypes significantly increased (102). Indeed, serotype 19A accounted for approximately 38% of cases in 2007 (103), which was consistent with data from other countries such as Canada (104), USA (105) and Spain (106). In addition, the introduction of PCV7 coupled with continued antibiotic use, also resulted in an increase in antibiotic resistance in non-vaccine serotypes of which 19A was the most prominent and highly resistant strain (107-110). Thus, considering multi-drug resistance associated with this particular serotype, serotype-replacement represents a significant health risk (111, 112). As a result, the PCV7 was modified to include additional serotypes (1, 3, 5, 6A, 7F, and 19A) in an attempt to induce broader spectrum coverage and to reduce invasive disease caused by these non-PCV7 serotypes. A study comparing immunization of healthy infants with the PCV7 and the 13-valent PCV (PCV13) reported that the PCV13 immunisation resulted in a significant reduction in nasopharyngeal colonization with the additional serotypes 1, 6A, 6C, 7F and 19A included in the vaccine formulation in comparison to that observed with the PCV7 (113). These findings also support other reports in France, the United Kingdom and the United States outlining the efficacy of the 13-valent PCV in reduction of these additional

serotypes (114-116). Although PCV13 is effective, it still does not protect against all pneumococcal serotypes and therefore does not resolve the problem of serotype replacement.

1.1.6.2 Experimental vaccines

Considering the serotype specific immunity generated by CPS-based vaccines, a vaccination approach that generates broad-spectrum immunity is required to reduce prevalence of invasive disease and consequently reduce use of antibiotics. Examples of such serotype-independent vaccines include protein vaccines and whole-cell vaccines.

Protein Vaccines

Protein vaccines are based on the possibility of using highly conserved purified proteins common to all serotypes. These proteins are T-dependent antigens and are therefore expected to induce long lasting immunity. Previous studies have tested the immunogenic potential of purified PspA, PspC, PsaA, Ply and PhtD (117-119). All of these proteins are important virulence factors that play a wide array of roles in pneumococcal pathogenesis. PspA is essential for inhibition of complement-mediated opsonisation (120, 121) and also has roles in preventing the antimicrobial activity of apolactoferrin (52). PspC is pivotal in adherence and invasion of host cells by pneumococci by binding to the polymeric immunoglobulin receptor, which is required for trancytosis of IgA across mucosal epithelial cells (122, 123). PhtD also has a role in attachment to epithelial cells although interactions still remain unclear (124-126). PsaA is involved in manganese uptake and resistance to oxidative stress (127, 128). Ply, as mentioned earlier, is a pivotal virulence factor that plays many significant roles contributing to pneumococcal pathogenesis (Section 1.1.4). Various combinations of these proteins rather than single antigens, have been shown to induce robust antibody responses and subsequently have proven to be protective upon pneumococcal challenge when administered either parenterally or via mucosal routes (117-119). Furthermore, in addition to the advantage of serotype-independence, protein vaccines can be produced at relatively low cost and in high quantity, which makes them quite promising vaccine candidates.

Whole-cell vaccines

Another approach towards generation of serotype-independent vaccines is employing pneumococcal whole-cells. Since the introduction of inactivated whole-organism vaccines by Almroth Wright in 1902, researchers have adopted this concept and have investigated the

effects of live-attenuated and completely inactivated whole-cell preparations as mucosal vaccines for *S. pneumoniae*. Employing bacterial whole-cells allows exposure of all antigens in their native biological conformation to promote induction of natural immune responses. In addition, the immunity induced by an array of antigens may be advantageous in comparison to protein vaccines or the PCVs, which comprise only a selected number of antigens.

1. Live attenuated pneumococcal vaccines: Live attenuated vaccines involve the attenuation of a strain by deletion or disruption of particular genes involved in virulence. Many studies have demonstrated the efficacy of these attenuated strains in mediating protective immunity against pneumococcal infections. For example, intranasal immunisation with a pep27 mutant strain (an autolysis-inducing factor involved in cell lysis) was shown to elicit serotype-independent protection against lethal intranasal challenge (129). In addition, Rosch and colleagues generated a live attenuated vaccine that involved the deletion of the *ftsY* gene, a signal pathway recognition component that is required for delivery of membrane and secretory proteins. This allowed pneumococci to colonise the nasopharynx without causing invasive disease. Similarly, intranasal immunisation was also shown to provide serotype-independent protection against multiple infectious models including sinusitis, pneumonia, otitis media and invasive pneumococcal disease (130). The approach of using nasopharyngeal colonization with live pneumococcal strains as mucosal vaccines has also been demonstrated in humans. Administration of a live encapsulated serotype 6B strain was shown to induce protective mucosal and systemic antibodies as well as preventing re-colonisation upon challenge (131). Although these attenuated strains have shown promising results, there is still a major safety concern for the use of live-attenuated vaccine strains in humans, especially those in immunosuppressed groups (132). Therefore, completely inactivated vaccine preparations are more desirable.

2. Inactivated pneumococcal whole-cell vaccines: Unencapsulated strains of pneumococcus have been utilised by many researchers to develop inactivated whole-cell vaccines. This allows maximum exposure of multiple pneumococcal antigens, particularly sub-capsular antigens for the induction of cross-reactive immune responses against multiple serotypes. Previous work by Malley and colleagues investigated the protective efficacy of whole-cell vaccines based on the unencapsulated Rx1 strain, which was modified to be autolysin deficient and to express a non–toxic derivative of Ply. Ethanol-inactivation of these modified pneumococcal whole-cells (whole-cell vaccine (WCV)) with cholera toxin as an adjuvant,

was shown to elicit significant protection against nasopharyngeal colonization and invasive disease following intranasal immunisation (133, 134). Protection with this vaccine was reported to be dependent on Th17 cell responses (85). Similarly, the effectiveness of an ethanol-killed pneumococcal strain, SPY1, defective in the capsule, teichoic acids and pneumolysin has also been investigated. Intranasal immunization with SPY1 and CT as an adjuvant was shown to provide serotype independent protection and reduce colonization (135). In these studies, both of these whole-cell vaccines utilized ethanol for inactivation of pneumococcal whole-cells. Considering the concerns with handling and disposal of large quantities of ethanol, Lu et al evaluated the immunogenicity of whole-cells inactivated by other chemical methods such as chloroform, trichloroethylene and β-propiolactone (136). Interestingly, these chemically inactivated WCV preparations were found to be more immunogenic than ethanol-killed preparations. In addition, chemical treatment with chloroform, trichloroethylene and β-propiolactone resulted in solubilization of approximately 15% of total proteins. However, these soluble components are retained and were proposed to contribute to immunogenicity (136). Despite the efficacy reported with these intranasal whole-cell vaccines, CT has not been approved as a safe mucosal adjuvant, which has lead to investigating the efficacy of whole-cell vaccines administered via parenteral routes. Vaccination subcutaneously with WCV inactivated by β-propiolactone with alum hydroxide as an adjuvant was shown to elicit serotype-independent protection and induce humoral (IgG) as well as Th17 mediated immunity (137-139). Thus, the protective efficacy reported with this vaccine prompted the development of the WCV into phase I clinical trials (ClinicalTrials.gov ID: NCT01537185) which are still in progress.

Nevertheless, considering that mucosal vaccination is desirable to defend against pneumococcal infection and carriage, development of a safe, intranasal whole-cell vaccine without the need for an adjuvant would be most optimal to defend against *S. pneumoniae.*

1. 2 Influenza A Virus

Influenza is a major respiratory pathogen, which is known to cause significant morbidity and mortality. It infects 3-5 million people worldwide resulting in approximately 250-500, 000 deaths per year (140). Influenza can infect people of all ages, but young children and the elderly are particularly susceptible. In addition, people suffering from underlying illnesses

such as respiratory failure, acute respiratory distress syndrome (ARDS), asthma and those with chronic lung or liver disease are at high risk of infection. Furthermore, people infected with respiratory bacterial pathogens such as *S. pneumoniae* are also very susceptible (141, 142).

Seasonal influenza epidemics refer to local outbreak of infection that prominently occur in the winter between November-April in the northern hemisphere and May-September in the southern hemisphere (143). In addition, introduction of novel viruses into the population through mechanisms such as antigenic drift and shift can result in the emergence of pandemics, i.e. global outbreaks of infection. Pandemics do not occur frequently, but when they emerge, they are potentially catastrophic, resulting in excessive mortality rates and a significant economic burden. Some of the major pandemics that have occurred since the 1900's include the Spanish Flu (H1N1) (1918–1920), Asian Flu (H2N2) (1957–1958), Hong Kong Flu (H3N2) (1968–1969) and more recently the swine Flu (H1N1) (2009) (144). The Spanish flu was the most devastating pandemic, as it resulted in 30-50 million deaths (145). In comparison, the 1957 and 1968 pandemics resulted in ~ 1 million deaths (146). Interestingly, geographical analysis of the Spanish flu pandemic showed that countries within Asia, sub-Saharan Africa and Latin America had significantly higher mortality rates compared to countries within Europe and North America (147, 148).

1.2.1 Biology and pathogenesis

Influenza is a member of the Orthomyxoviridae family. It is an enveloped negative-strand RNA virus which is comprised of 8 genome segments that encode surface glycoproteins (hemagglutinin (HA) and neuraminidase (NA)), matrix proteins (M1 & M2), as well as internal proteins such as nucleoprotein (NP), basic polymerase protein 1 and 2 (PB1, PB2), and acidic polymerase (PA) (142, 149) (Figure 1.2). There are 3 classes of influenza viruses (A, B and C), based on antigenic differences in their NP and M proteins. Influenza A viruses can infect both avian and mammalian species, whereas Influenza B and C viruses are essentially restricted to humans (141). It is viruses within this A class that are responsible for the vast majority of pandemics that have occurred throughout history. This is in contrast to B and C class viruses, which have limited outbreaks and are normally associated with low level sporadic disease (150). Influenza A viruses can be further subtyped based on the differences in surface glycoproteins HA and NA; there are 16 known subtypes of HA and 9 subtypes of

NA identified to date (151). This review will focus on the biology of influenza A class viruses.

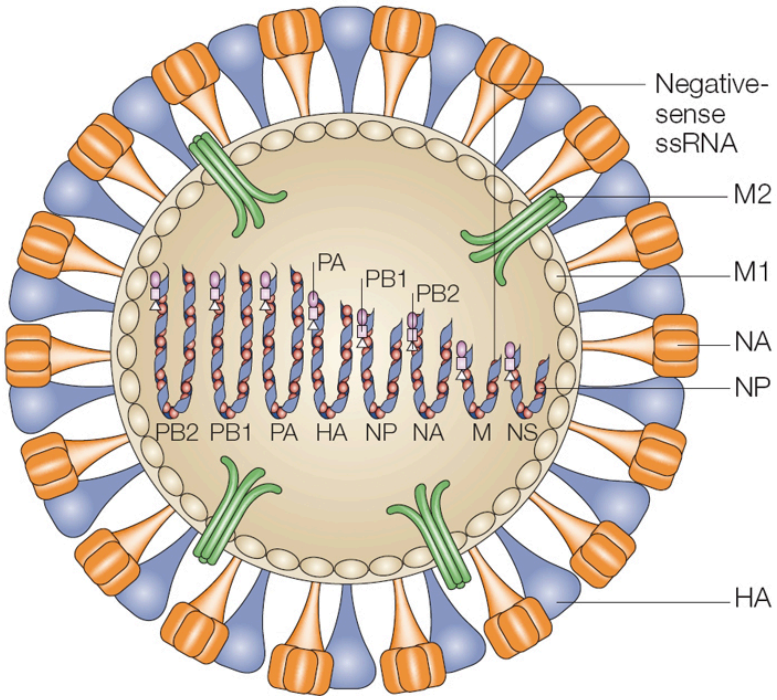


Figure 1.2: Influenza structure (152)

Influenza A causes acute inflammation within the upper and lower respiratory tract that results in severe epithelial destruction (142). Infection results in typical symptoms such as fever, muscle aches, fatigue, runny nose and sore throat, which last 7-10 days (153). Influenza has been shown to enter the host cell via receptor-mediated endocytosis upon binding of the HA surface protein to sialic acid receptors (α-2,6-linked sialic acid for human strains) located on cell surfaces of epithelial cells in the upper respiratory tract (154, 155). Upon internalization of the virus, the M2 protein, a low pH gated proton channel, lowers the pH of the virion to facilitate fusion of viral and endosomal membranes. This leads to the release of viral RNA segments into the cytoplasm to be trafficked to the nucleus (156). Subsequently, transcription and replication of the viral genome is mediated by the viral RNA polymerase complex, which comprises PA, PB1 and PB2 (157, 158). Each influenza viral RNA segment is coated with NP to form a ribonucleoprotein (RNP). After replication, newly synthesized RNP complexes are exported from the nucleus to the cytoplasm by the nuclear export protein (NEP) and M1. NA then cleaves sialic acid residues from

sialyloligosaccharides, which allows the release of progeny virus from infected cells for dissemination within the host (159, 160).

Like all pathogens, influenza also possesses virulence factors such as non structural protein 1 (NS1) and PB1-F2, which mediate evasion of host anti-viral defence mechanisms. NS1 is pivotal for inhibiting IFN-I production. It interferes with RIG-I signalling, which subsequently prevents induction of interferon-stimulated genes (161). Alternatively, PB1-F2, translated from an alternative reading frame in the PB1 gene, has been shown to interact with mitochondrial membrane proteins, adenine nucleotide translocator 3 (ANT3) and the outer mitochondrial membrane voltage-dependent anion channel 1 (VDAC1). Interaction with these proteins has been shown to cause permeabilization of the mitochondria which in turn promotes apoptosis of infected cells (162).

1.2.2 Immunity

1.2.2.1 Innate immunity

Various components of the innate immune system play pivotal roles in recovery from influenza infection. Upon infection of respiratory epithelial cells or immune cells such as DCs, viral components are recognized by PRRs such as TLRs and retinoic acid–inducible gene (RIG)–like receptors. During infection, TLR7 and RIG-I recognize the single stranded RNA genome, whereas upon replication, double stranded RNA is recognized by TLR3 (163-165). Signaling through these PRRs promotes NF-κβ activation and production of pro-inflammatory mediators, particularly IL-6, IL-12, TNF-α and IFN-I (166). IFN-I produced predominantly by macrophages and plasmacytoid DCs, is essential for stimulating the expression of IFN-stimulated genes which promote anti-viral defences to limit viral replication and dissemination throughout the host (167). Signaling through these PRRs also promotes the production of chemokines for recruitment of effector immune cells, such as macrophages, neutrophils and NK cells to the airway to mediate phagocytic clearance of virus-infected cells (168, 169). As mentioned earlier, influenza possesses the virulence factor NS1, which is able to evade the innate immune response by inhibiting the production of IFN-I through interfering with RIG-I signalling, as well as binding to protein kinase R (PKR) (165, 170, 171).

Activation of the inflammasome complex has also been demonstrated to be essential for survival following influenza infection (172). Single stranded RNA, as well as viral proteins

M2 and polymerase PB1-F2, have been shown to activate the NLRP3 inflammasome. Activation ultimately leads to the release of major pro-inflammatory mediators IL-18 and IL-1β (173-175). Inflammasome activation can promote pyroptosis of infected cells (176) and has been shown to be important in tolerance to infection through mechanisms such as tissue repair (175). This is contrary to the direct anti-viral resistance observed with induction of IFN-I.

1.2.2.2 Adaptive immunity

Antibody responses

Various arms of the adaptive immune response play critical roles in the defence against influenza infection including humoral responses. In general, antibody responses generated against the influenza surface proteins are critical for host recovery, as demonstrated in many studies using B cell deficient mice (177, 178). Antibodies against the receptor binding sites of the HA glycoprotein are most important in neutralizing the virus, as it prevents attachment of virions to host cells (179). In addition, antibodies against other surface viral proteins NA and M2 have also been demonstrated to contribute to protection against live virus challenge (180-182). All classes of antibodies (IgG, IgA and IgM) can be detected following influenza infection, but IgA has been found to be the principle isotype within the respiratory tract that provides superior protection (183). Generally, due to antigenic changes that occur in the surface proteins (HA and NA), antibodies directed against the globular heads of these epitopes are only effective in preventing re-infection with the same strain and do not accommodate for antigenic variation that generates drift variant virus strains (184, 185). However, there have also been reports of cross-reactive antibodies that mediate protection against heterosubtypic challenge, as observed following transfer of anti-HA IgA sera into naïve mice (186). In particular, these cross-reactive antibodies have been shown to be specific for conserved stalk domains of HA (187).

T cell immunity

T cell immune responses play an important role in recovery from influenza infection. $CD8^+$ T lymphocytes (CTLs) recognize highly conserved viral epitopes from internal viral proteins and have been demonstrated to be essential for clearance of virus-infected cells (188, 189). Upon recognition of MHC class I-antigenic peptide complexes on viral-infected cells, CTLs cause cell lysis by release of cytotoxic mediators such as perforin and granzyme (190, 191).

Studies have demonstrated the importance of CTLs during influenza infection, as their cytotoxic effects have been shown to mediate recovery in mice devoid of antibodies and mature B cells (192). Unlike strain-specificity associated with humoral responses, CTL responses are highly advantageous within vaccine design as they are cross-reactive against drifted as well as heterosubtypic viruses. Furthermore, $CD4^+$ T cells are also vital in the host's defence against influenza and have been shown to be protective independently from B cells and $CD8^+$ T cells (193). Interestingly, like $CD8^+$ T cells, $CD4^+$ T cells in the lung have also been shown to directly kill influenza-infected cells in a perforin-dependent manner (194). In addition, follicular $CD4^+$ T (Tfh) cells located within the lymph nodes and circulation are essential for providing help to B cells for activation/differentiation to promote the development of class switched antibodies by viral specific B cells and plasma cells (195, 196). Influenza is also associated with the polarization of Th1 cells, which are important for secreting cytokines such as IFN-γ, TNF-α and IL-2 (197). Th1 signature cytokine IFN-γ is pivotal for promoting isotype switching to IgG2a and IgG3 and for the recruitment of innate immune cells such as NK cells (198-200), whereas IL-2 secretion has been shown to be important in enhancing the proliferation of CTLs (201).

Another specialised T cell memory subset known as tissue resident memory (TRM) cells has been reported to be important for faster secondary responses upon a secondary infection. TRM cells are non-circulating memory cells that persist for long periods in peripheral tissues such as the lung (202), skin (203), gut (204) and brain (205). These cells can respond rapidly to antigen at the site of infection and are completely independent of T cells from the circulatory pool. They have a distinct phenotype from T effector and T central memory subsets that have been defined by upregulated expression of common markers such as CD69, CD103 and CD11a (203, 206, 207). In particular, it has been shown after influenza infection that lung TRM cells express CD103 (208). Importantly, both $CD4^+$ and $CD8^+$ TRM cell subsets have been shown to be essential for mediating optimal protection upon influenza infection (209, 210). Therefore, given the superiority of TRM responses in comparison to circulating memory, current vaccination strategies are attempting to generate vaccines that establish these local memory responses.

1.2.3 Influenza Vaccines

Influenza virus has caused ever-evolving problems for vaccinologists due to the continuous emergence of new strains and the limited scope of cross-protective immunity within current vaccine preparations. Pandemic influenza A virus infections are known to be associated with high degree of morbidity and mortality worldwide (142, 211). The best method of prevention and control of influenza infection is vaccination (140). Creating a universal influenza vaccine is very challenging due to high frequencies of mutations that occur in the structural genes, through mechanisms that include antigenic-drift and -shift (185). This is due to the low fidelity of the RNA-dependent RNA polymerase during replication, which introduces point mutations in the surface glycoprotein coding sequences. As a result of these antigenic variations, previously acquired immunity is often ineffective, allowing the newly emerged virus strains to evade the hosts' immune recognition upon re-infection. This results in epidemic and pandemic outbreaks (155, 212). Therefore, influenza vaccines require annual updating to match the newly arising strains that cause seasonal endemics (142).

The current licenced influenza vaccines are multivalent and comprise components of influenza A: H1N1, H3N2 and influenza B strains. These vaccines are separated into 2 classes: inactivated and live attenuated. Inactivated vaccines are administered parenterally and are either split virus, whole virions, subunit (purified glycoproteins) or recombinant HA based vaccines. Inactivation methods involve the disruption of the virus using chemicals such as β-propiolactone (213) and ether (214). Consequently, these vaccines mainly induce antibody responses against the surface glycoproteins (HA & NA), but lack the ability to elicit strong cross protective T cell responses against conserved internal proteins (214-216). Although inactivated vaccines are safe, they are not capable of providing full protection against newly emerging influenza strains and thus have prompted the development of live attenuated influenza vaccines (LAIV). LAIV are currently licenced for human use and are administered through the intranasal route. These vaccines also contain a mixture of influenza strains but they are attenuated so they can mimic natural infection without causing significant clinical infection. LAIV are heat sensitive, which allows them to replicate only in the nostrils but not in lower regions of the respiratory tract such as the lungs. Examples of currently licenced intranasal vaccines for influenza are FluMist® and NasoVac®. FluMist® comprises influenza A strains H1N1 and H3N2 as well as 2 influenza B strains. FluMist® has been approved for use in healthy people from 5-49 years. Alternatively NasoVac®, only contains

the pandemic A/California/7/2009 (H1N1) strain and is recommended for adults, the elderly and children over the age of 3 (217, 218). These strains can induce strain specific IgG and mucosal antibody responses. In addition, they induce effective cross protective T cell responses, which renders them highly efficient against antigenically drifted strains of influenza (219, 220). Despite the efficacy reported with LAIV, there are still concerns regarding the ability of these vaccines to revert to their wild type virulent form. There have also been reports of adverse side affects such as respiratory symptoms (cough, runny nose, sore throat and wheezing). In particular, in some patients wheezing has been reported to last up to 42 days, which prevents administration of this vaccine to patients with unstable asthma (221). Furthermore, problems with efficacy in the elderly and restriction of age groups that can receive these LAIV increases the demand for a universal vaccine that can protect all age groups against multiple strains of influenza, particularly upon emergence of a pandemic strain. In fact, since the proofing of this thesis, the Advisory Committee on Immunization Practices (ACIP) have recommended that FluMist® not be used during the 2016-2017 influenza season.

Recently, gamma-irradiation has been utilised as a method of inactivation to develop whole inactivated virions for intranasal administration. Previously published data illustrate that gamma-irradiated influenza vaccine (γ-FLU) is a promising mucosal vaccine candidate that induces cross-protective immunity (222-224) (Discussed below in Section 1.7.1).

1.3. Co-infection with Influenza and *S. pneumoniae*

Influenza-induced susceptibility to secondary bacterial pneumonia is well documented and it is responsible for the excess mortality that occurs during influenza pandemics. The 1918 pandemic was reported to be associated with the infection of 500 million people worldwide and more than 50 million deaths (225, 226). Importantly, *S. pneumoniae* was the most common bacterial pathogen recovered from the sputum, lung and the blood of infected patients. Fortunately the subsequent introduction of antibiotics and influenza vaccines significantly reduced mortality rates during the Asian flu (1957-58) and the Hong Kong Flu (H3N2) (1968–1969) pandemics relative to the 1918 pandemic (225, 227, 228). Nevertheless, there still remains a very significant morbidity and mortality rate associated with newly emerging pandemics. This is evident from the more recent influenza pandemic that occurred

in 2009, which resulted in approximately 200,000 deaths. Similarly, *S. pneumoniae* and *S. aureus* were the common bacterial pathogens associated with bacterial super infection, which resulted in substantially higher numbers of deaths. Overall, this signifies the continuous global threat of lethal synergism between these pathogens (229, 230).

1.3.1 Mechanisms underlying synergism

Primary infection with influenza induces many factors that enhance susceptibility to pneumococcal infection (Figure 1.3). Recent reports have described a number of mechanisms underlying the synergism between the two pathogens. Firstly, alterations in lung physiology represent the major factor responsible for facilitating the entry of invading bacteria into the respiratory epithelium. Severe epithelial cell destruction caused by influenza in the lung, in conjunction with loss of epithelial cell reproliferation and repair, exposes binding sites and facilitates bacterial attachment (231, 232). In addition, the cleaving of sialic acid receptors by the influenza glycoprotein NA increases the exposure of adherence receptors, which also promotes bacterial colonization (233). In particular, expression of PAFr is upregulated which is one of the major receptors that facilitate pneumococcal cell adherence to host cells (234). Furthermore, the epithelial ciliary function is also compromised, leading to a decrease in the mechanical clearance of bacteria (235).

Apart from changes in the lung physiology, there are also a number of chemical mediators induced by influenza, which further promote bacterial infection. Expression of the viral cytotoxic accessory protein PB1-F2 has been reported to significantly enhance inflammatory responses and cell death, which subsequently increases the severity of pneumococcal pneumonia (236, 237). In addition, high levels of pro-inflammatory cytokines induced during influenza infection such as IFN-I have been reported to downregulate neutrophil chemoattractants CXCL1 and CXCL2, thereby impairing neutrophil recruitment for bacterial clearance (238). Moreover, high IFN-I levels have also been shown to attenuate the production of IL-17 by γδ T cells, which also interferes with neutrophilia (239). In particular, it has been noted that IFN-I dependent production of IL-27 is associated with suppression of these IL-17 producing γδ T cells (240). Other cytokines such as IFN-γ and IL-10 have also been implicated in enhanced susceptibility to secondary bacterial pneumonia. IL-10, known to be important for suppression of neutrophil functions such as degranulation (241), was shown to play a significant role in reducing neutrophil-mediated pneumococcal clearance

(242). Moreover, high IFN-γ levels induced during influenza infection have been shown to downregulate the expression of the scavenger receptor MARCO on alveolar macrophages, thereby suppressing bacterial uptake and killing by phagocytosis (243). In addition, previous viral infection was shown to desensitize lung alveolar macrophages to TLR ligands and consequently reduce NF-κB activation and chemokine production, significantly affecting neutrophil recruitment (244). Influenza infection also depletes airway-resident alveolar macrophages, which affects early surveillance and phagocytic clearance of pneumococci, consequently enhancing the susceptibility to bacterial infection (245).

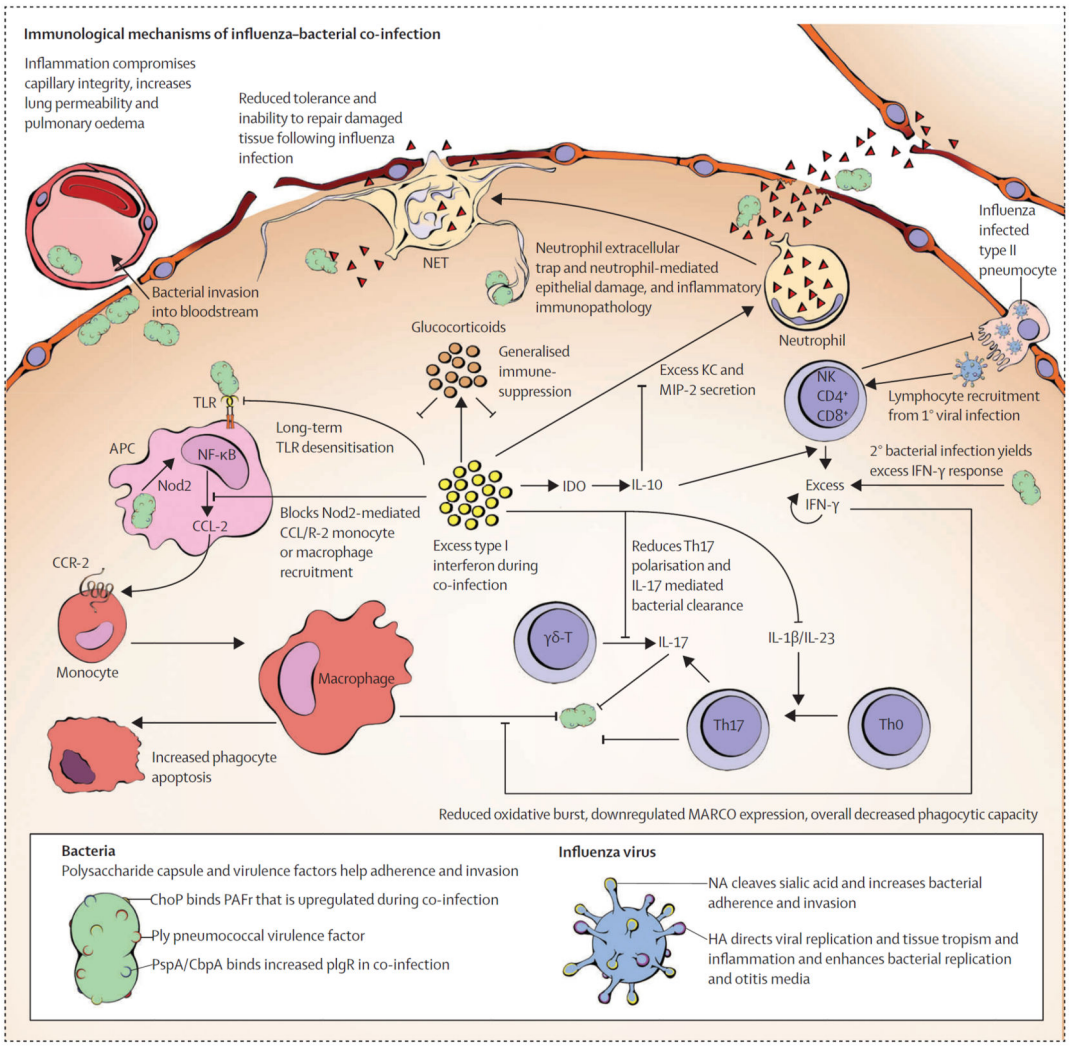


Figure 1.3: Mechanisms underlying influenza and bacterial co-infection (246).

Overall, a combination of these physical and chemical factors facilitates pneumococcal colonization and dissemination throughout the host. Although multiple mechanisms have been reported, there still remain many unknown factors regarding the mechanisms that result in enhanced susceptibility to bacterial infection. The cellular immune defence mechanisms

against *S. pneumoniae* and influenza infections are disparate. Antibody-dependent opsonisation and neutrophil mediated phagocytosis are responsible for effective clearance of the bacteria, whilst cytotoxic $CD8^+$ T lymphocytes are essential for clearance of virus-infected cells. However the innate immune mechanisms elicited to control these infections are quite similar. In addition to the physical barriers as the usual first line of defence (i.e. mucus, ciliary beating), both *S. pneumoniae* and influenza are capable of signalling through TLRs in order to promote the production of cytokines and recruitment of immune effector cells. Considering the overlap in the induction of pro-inflammatory mediators, it is reasonable to expect that the two pathogens have synergistic effects within the host, which further complicates the process of controlling co-infection.

1.3.2 Vaccination strategies against co-infection

As discussed previously, vaccines are currently employed to protect against invasive disease caused by influenza and *S. pneumoniae* infections individually. In line with this, researchers have reported the positive impact of single vaccination against either pathogen on reducing the effects of co-infection. For example, in Africa, vaccination with a 9-valent pneumococcal conjugate vaccine was shown to reduce pneumonia associated with influenza by 45% (247). Similarly, a study evaluating the frequency of bacterial otitis media cases in young children under the age of 5, reported that vaccination with the trivalent inactivated influenza vaccine (Fluarix) reduced the incidence of acute otitis media by ~50% (248). In recent years, scientists have also investigated the synergistic effect of dual vaccination at separate administration sites on the overall outcome of co-infection. A large study conducted in the USA in elderly persons with chronic lung disease reported that patients receiving both an *S. pneumoniae* and an influenza vaccine had a reduced incidence of hospitalization for pneumonia (~63%) and an 81% reduction in the risk of death, in comparison to patients receiving single immunisation (249). A large study in Sweden focused on elderly persons also reported a 29% and 44% reduction in overall hospital admissions for pneumonia and IPD respectively, when patients received both vaccines. These values were higher in comparison to patients receiving the pneumococcal vaccine alone. In addition, there was a 37% reduction in influenza hospitalization observed in patients receiving both vaccines in comparison to 26% observed in patients that only received the influenza vaccine (250). A study conducted in Spain also found that vaccination with both the PPV23 and influenza vaccines reduced influenza hospitalization by ~ 81%, which was significantly higher than the

41% reduction observed with patients only vaccinated with the PPV23 vaccine (251). The additive effect of dual vaccination on the outcome of influenza or pneumonia infections has been further demonstrated in studies conducted in Taiwan (252) and Japan (253).

Taken together, previous studies illustrate the effectiveness of dual vaccination regimes in protection against both of the pathogens to limit the effects of co-infection. However, despite the success observed with single vaccination regimes, lack of optimal vaccines that offer broad-spectrum serotype-independent and heterosubtypic protection against *S. pneumoniae* and influenza respectively, still remain a significant global problem (98, 142). Thus, the prospect of designing a single vaccine to protect against both pathogens would be highly desirable. Katsura and colleagues developed a bivalent vaccine against *S. pneumoniae* and influenza which comprised of a replication incompetent hemagglutinin knock out influenza strain that expressed the antigenic region of pneumococcal PspA. Interestingly, this vaccine was shown to protect against lethal challenge with individual pathogens (254), but the efficacy of this vaccine against co-infection was not reported. Aside from this report, there are very limited studies evaluating the effectiveness of incorporating antigens from both pathogens as a single vaccination approach to protect against influenza and *S. pneumoniae* individual disease in conjunction with their lethal synergism.

1.4 Combination vaccines

Combination vaccines have been investigated since the early 1940's as a means to protect against multiple diseases. This strategy was initially employed to minimize the number of injections a child must receive in an attempt to simplify childhood immunization schedules. Other advantages include fewer visits to the health practitioner, reduced vaccination administration costs and higher coverage rates (255). In addition, considering that the number of vaccines available for immunization is increasing over the years, combination vaccines will be essential to minimise the number of injections required.

Within combination vaccine design, the immunogenicity of co-administered vaccine antigens is generally intended to be equivalent or higher than administration of the vaccines separately. It is also important that immune responses elicited by each of the vaccine antigens does not inhibit or interfere with the quality of immunity induced by co-administered

antigens. Diphtheria-tetanus-acellular pertussis (DTaP) and measles, mumps and rubella (MMR) combined vaccines are among the few combination vaccines that are effective in the clinical setting (256, 257). DTaP is comprised of inactivated toxins and surface antigens from bacterial pathogens *Corynebacterium diphtheria*, *Clostridium tetani* and *Bordetella pertussis*. Many studies have shown the protective efficacy of the vaccine and have reported sufficient antibody titres to each of the antigens (69, 258). The DTaP vaccine was shown to elicit Th2 polarized immunity associated with elevation of cytokines IL-4, IL-5 and IL-13, as well as enhanced levels of IgE (84, 259). In particular, type 2 antibody responses (IgG1) were reported to be most effective at neutralising the diphtheria toxin (260). Alternatively, the MMR vaccine, first licenced in the early 1970's, is a very effective combination vaccine, which comprises live attenuated measles, mumps and rubella viruses. Similarly, combination of this trivalent vaccine was shown to induce high titres of antibodies against each of the included viruses (261). It was also reported to induce good cellular immune responses, which is important for intracellular pathogens. In particular, the measles component induced a strong Th1 response, which was associated with an increase in NK and $CD8^+$ cells (262).

Over the years, scientists have explored the possibility of combining additional antigens to existing vaccine formulations and subsequently have developed combination vaccines containing both viral and bacterial antigens. A good example is the hexavalent vaccine (DTaP-IPV-Hep B-Hib), which involves the addition of inactivated poliovirus (IPV), hepatitis B virus (Hep B) and *Haemophilus influenzae* type b (Hib) to the pre-existing DTaP vaccine. This elicits optimal immunity against 6 of the major childhood diseases. This combination vaccine has been shown to induce immunity against all vaccine antigen components (263, 264). Although this vaccine contains a mixture of viral and bacterial antigens, there are currently no combination vaccines that comprise whole virions and whole bacterial cells.

1.5. Intranasal Vaccination

Many viral and bacterial pathogens enter through mucosal membranes such as the respiratory, intestinal or urinogenital tracts. The mucosa has a highly developed immune system and many researchers have investigated the possibility of using mucosal immunization as a means of generating local as well as systemic immune responses against

mucosal pathogens (219, 265). For common respiratory pathogens such as influenza and *S. pneumoniae*, intranasal delivery of vaccine antigens into the nasal mucosa is advantageous for generating antigen-specific mucosal immune responses. Normally, during intranasal delivery, antigen uptake occurs at mucosal lymphoid follicles located within the nasopharynx. This is referred to as the nasopharynx-associated lymphoid tissue (NALT) in rodents and the Waldeyers ring in humans (the adenoid and tonsils) (266-269). This inductive site is composed of specialised follicle-associated epithelium that contains microfold cells (M cells) as well as antigen specific T and B cells. M cells are specialized epithelial cells that act as membrane transporters. They readily sample antigens from the lumen of the nasal mucosa and deliver them to intraepithelial antigen-presenting cells (B cells, DC's and macrophages) for processing and presentation. This subsequently leads to the local activation and expansion of effector memory B and T cells (270, 271). B cells transform into plasma cells, which generate specific mucosal IgA and IgG antibodies that are typically important for preventing pathogens from attaching to the nasal epithelium (272, 273). Alternatively, DCs migrate to B cell and interfollicullar T cell zones to present antigens to naïve T cells, which promotes cellular immunity (274). The nasal mucosa is highly vascularised which allows entry of these activated T and B cells into the circulation mediating systemic immune responses (275) (Figure 1.4).

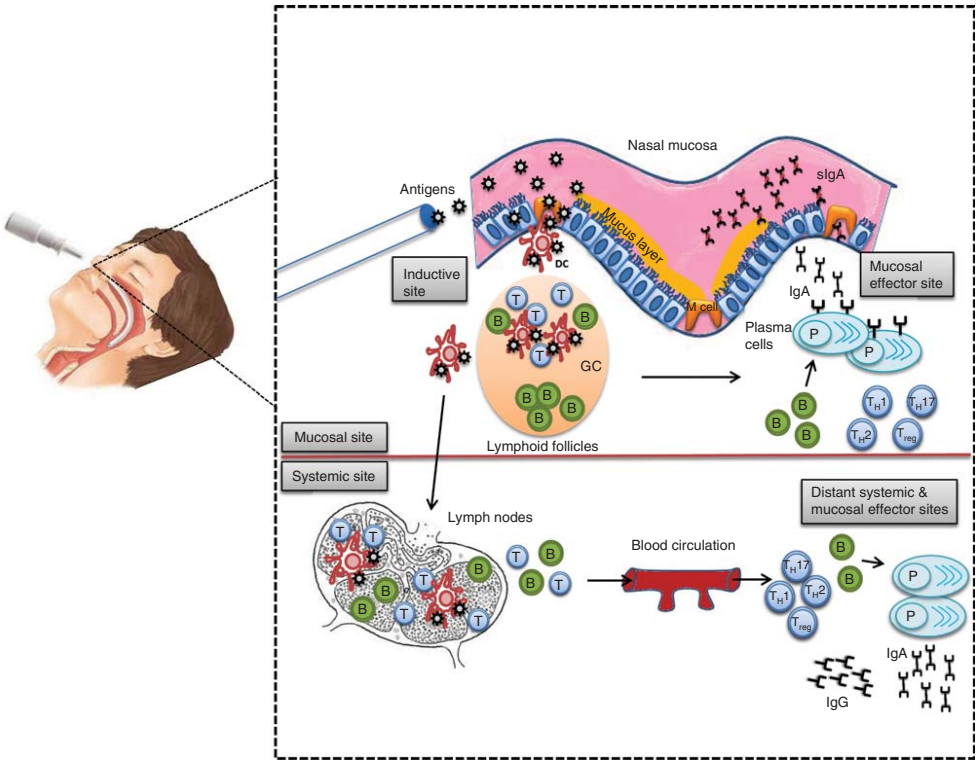


Figure 1.4: Intranasal administration and induction of mucosal immune responses (219).

Intranasal immunisation as a means to induce appropriate immunity for mucosal pathogens is highly advantageous due to the fact that it is relatively safe, easy, painless, needle free and a more publicly acceptable vaccination approach. It also decreases the risk of disease transmission through the use of syringes. Intranasally delivered vaccines approved for human use (FluMist® and NasoVac®) are based on live attenuated influenza strains, which mimic natural infection, but do not produce symptoms other than mild rhinitis due to reduced virulence.

1.6. Adjuvants for intranasal delivery

Although the currently approved intranasal vaccines (live attenuated vaccines) are highly efficacious, there are still concerns that these live attenuated strains might revert to their virulent form, compromising overall safety of these vaccines. Therefore, scientists have shifted their focus towards developing inactivated intranasal vaccines using either subunit or split viruses and also purified antigens. Although they are considerably safer, some of these vaccines still remain poorly immunogenic. The goal for mucosal immunization is to generate a vaccine that upon delivery is sustained within the nasopharynx for a sufficient period of time to ensure the appropriate induction of long lasting immune responses. Therefore, adjuvants have been employed for delivery of nasal antigens and studied extensively as an approach to enhance immunogenicity towards the co-administered vaccine antigens. They target specific arms of the immune system to tailor the most appropriate immune responses for the desired antigen. Common intranasal adjuvants investigated to date include enterotoxins such as cholera toxin (CT) and heat labile toxin (LT), Toll like receptor (TLR) agonists and natural killer T cell (NKT) agonists. Each of these adjuvants will be discussed briefly below.

1.6.1 Enterotoxins

Enterotoxins produced by *Vibrio cholerae,* such as CT, and LT from *Escherichia coli* are the most experimentally used mucosal adjuvants. They are the most potent mucosal adjuvants and have been reported to enhance both systemic and mucosal antibody responses against co-administered antigens (276). These toxins comprise an A subunit which has ADP-ribosylating activity and a B subunit which mediates binding to GM1-ganglioside receptors on the surface of mammalian cells (277). Although, many studies have proposed mechanisms

by which enterotoxins such as CT enhance immunogenicity, the precise mechanisms are still not fully understood. In particular, CT is known for its superior ability to augment mucosal IgA responses, through stimulating B cell isotype switching to IgA (278). Furthermore, it has been shown to increase the permeability of the transepithelial cell layer, which ultimately leads to greater absorption of the vaccine antigen into the NALT (279). As a result, CT has been reported to enhance antigen processing and presentation through its ability to upregulate MHCII and co-stimulatory molecule expression on APCs. In addition, it has also been shown to increase the expression of chemokine receptors, particularly CCR7 and CXCR4 on mature DC's, which has been proposed to accelerate migration to secondary lymphoid organs for presentation to T cells (280, 281). Moreover, CT favours the induction of Th2 responses, which are associated with high levels of IgG1 and release of cytokines Il-4, IL-5 and IL-10 (281, 282). However, CT has also been reported to induce a Th17 dominated response to bystander antigens (283).

The potent stimulatory effects of CT have been documented in many studies when administered through mucosal routes with a range of viral and bacterial antigens. Specifically for *S. pneumoniae*, CT has been employed as an effective adjuvant for protein antigen vaccines (117, 284, 285), as well as for pneumococcal whole-cell vaccines (133, 286) to enhance immunogenicity and protective efficacy against pneumococcal challenge. Although enterotoxins are superior adjuvants, they have been reported to penetrate into the CNS via the olfactory nerve, increasing the potential for neuronal damage (287). Indeed, reports of adverse effects in humans such as facial nerve palsy, have prevented the clinical application of these adjuvants (288).

1.6.2 TLR agonists

Most immunostimulatory adjuvants have been designed to potently stimulate innate signalling pathways through PRRs such as TLRs and cytosolic receptors (289). Activation of these receptors results in the recruitment of adaptor molecules such as MyD88 or TRIF, which ultimately leads to the activation of essential transcriptional programs involving key transcription factors such as NF-κB, IRF-3 and IRF-7. This ultimately leads to the induction of genes encoding essential cytokines such as IFN-I, that further promote the induction of inflammatory cytokines, anti-viral enzymes and co-stimulatory molecule expression important in priming the immune response (290). Many studies have evaluated the efficacy

of TLR agonists as mucosal adjuvants. For example, cytosine phosphate guanosine oligodeoxynucleotides (CPG ODN) resemble sequences that are found in bacterial DNA which are commonly recognized by TLR-9 (291). CPG ODN has been demonstrated to be effective as an adjuvant for inducing B cell differentiation, proliferation and promoting isotype class switching (292). It has also been shown to upregulate expression of MHCII and co-stimulatory molecules for augmenting antigen presentation (293). In addition, CPG ODN-mediated signalling promotes Th1 dominated responses associated with enhanced IFN-γ, IL-12, class switching to IgG2a and production of CTL responses (294, 295). In particular, studies with a formalin-inactivated influenza virus have shown that intranasal administration with CPG ODN effectively enhanced influenza-specific systemic and mucosal antibody responses (296).

The TLR4 agonist, monophosphoryl lipid A (MPL) (a detoxified form of lipid A from *Salmonella minnesota* R595) is also widely used experimentally and is one of the clinically approved adjuvants for human use. It is commonly incorporated within adjuvant formulations containing liposomes and alum. MPL is a licenced adjuvant component within the hepatitis B vaccine Fendrix® and the Human papilloma virus (HPV) vaccine Cervarix® (297, 298). The effectiveness of MPL has been attributed to its ability to elicit cytokine cascades upon macrophage activation such as TNF-α, IL-2 and IFN-γ, which promote the development of Th1 responses (299). The intranasal adjuvant activity of MPL has also been demonstrated with antigens from influenza, hepatitis B and *Clostridium tetani.* MPL was shown to enhance levels of IgG2a to all antigens, highlighting the strong induction of type 1 antibody responses (300).

Imidazoquinolines, modified synthetic ssRNA that are recognised by TLR7 and TLR8, have also been reported to have adjuvant activity (301). Intranasal delivery of an imidazoquinoline-based TLR7 agonist, gardiquimod (GARD) with Norwalk virus-like particles was shown to induce profound antigen-specific humoral responses. However, in contrast to CPG ODN and MPL agonists, this was associated with strong polarization of Th2 responses as indicated by higher IgG1 levels. It was also reported to enhance mucosal IgA responses in gastrointestinal, respiratory, and reproductive tract samples (302).

Finally, considering that IFN-I induction is pivotal upon TLR activation, previous studies have also demonstrated the beneficial influence of exogenous IFN-I on the magnitude of

immune responses to mucosal inactivated vaccines. Combination of IFN-α/β with an intranasally delivered influenza subunit vaccine was shown to enhance the antibody levels and increase the survival rates of mice compared to vaccination without IFN-I (303).

1.6.3 Natural killer T (NKT) cell agonists

Another popular adjuvant that has been utilized to enhance immunogenicity of mucosal vaccines is invariant natural killer T (NKT) cell agonists such as α-galactosylceramide (α-GalCer). NKT cells are a distinct population of αβ TCR^+ T cells, which have similar characteristics to natural killer (NK) cells. However, they express a restricted TCR repertoire that recognises glycolipids such as α-GalCer (a glycolipid extracted from marine sponges), which bind to the MHC class I like molecule CD1d (304, 305). They secrete large amounts of cytokines such as IL-4 and INF-γ that can significantly modulate the immune response towards either Th1 or Th2 responses (306-308). NKT cells are also important for the activation of immune subsets such as NK cells, macrophages and B cells. In particular, NKT cells have been shown to be involved in maturation and proliferation of B cell memory responses and have been demonstrated to enhance CTL activity, as well as mediating direct cell lysis through Fas and perforin-dependent mechanisms (309-312). The effectiveness of α-GalCer as an intransal adjuvant has been associated with strong induction of systemic and mucosal antibody responses as well as enhancement of CTL responses (313). Furthermore, protective immune responses have been observed in a NKT-cell mediated influenza vaccine, whereby immunization of mice with a formalin-inactivated whole virion vaccine with α-GalCer exhibited higher secretory IgA responses and provided heterosubtypic protection against influenza challenge, in comparison to mice vaccinated without α-GalCer. Furthermore, this study also showed that immunization with α-GalCer and an HA influenza vaccine was associated with a localized increase in the number of NKT cells (314).

1.6.4 Delivery systems

Generally antigens have minimal affinity for the nasal epithelium and do not have a long retention time before they are removed by mucociliary clearance. In addition, other factors such as nasal enzymes and high mucus turnover hinder the effective absorption of these vaccine antigens into the nasal mucosa. Therefore to overcome these obstacles, in addition to using adjuvants, different delivery systems have been explored to act as carriers to deliver antigens to target host cells to facilitate longer exposure time and absorption into the NALT.

These include formulation with chitosan, polylactic acid, liposomes and virosomes, but these components will not be discussed in great detail here, as they have been extensively reviewed previously (265, 273).

Regardless of the specific mechanisms whereby adjuvants enhance immunity, a good adjuvant should be non-toxic, induce good cellular as well as humoral responses tailored to the pathogen of interest, and produce high quality, long-lasting memory responses.

1.7. Gamma Irradiation

Gamma irradiation involves the use of high frequency electromagnetic radiation referred to as 'gamma rays'. Ionizing radiation generated from a cobalt 60 source has a very high penetrative strength which allows it to go through most biological and non-biological materials in contrast to other forms of irradiation such as UV (315). Thus, it has been used world-wide as a sterilisation method for food (316) and pharmaceuticals (317) and as a means of inactivation of highly infectious agents such as Ebola, Marburg and Lassa viruses (318). Gamma-irradiation can inactivate biological material by two mechanisms; direct and indirect. The direct mechanism is the dominant pathway of inactivation and involves the direct interaction of gamma-rays with nucleic acids of the organism, which results in genome disruption via strand breakage and base mutations but with minimal interactions with structural proteins. Alternatively, the indirect mechanism involves the interaction of gamma-rays with water molecules and oxygen that are present in the solution/extracellular milieu. This results in the production of short-lived free radicals and reactive oxygen species, which can cause damage to proteins (319, 320). There are also several factors that can affect the process of inactivation by gamma-irradiation, such as the target size, the complexity of the genome (single or double stranded), the composition of the irradiated materials, and the irradiation temperature (320-324).

1.7.1 Gamma-irradiation as an inactivation method for vaccines

Although gamma-irradiation typically has been used as a safe approach for decontamination of food, in recent decades it has also been employed as an inactivation technique for the development of vaccines. Generally, viral pathogens have been shown to be more resistant to gamma-irradiation due to their smaller size and genetic composition, in comparison to bacteria, parasites and fungi (70, 325, 326). Many studies have reported the efficacy of

gamma-irradiation as an inactivation technique to destroy infectivity whilst still maintaining antigenicity for a number of viral pathogens. For example, gamma-irradiated Venezuelan Equine Encephalitis virus was shown to induce high levels of neutralizing antibodies and protection against lethal viral challenge in guinea pigs (327). Similarly, vaccination with gamma-irradiated Ebola Virus was shown to induce high CTL responses and provided protection against challenge in rodents, but not in primates (328).

Recent studies have explored the effectiveness of gamma-irradiation for the development of a cross-protective influenza vaccine. Gamma-irradiated influenza whole virions (γ-FLU) has been shown to elicit superior immunogenicity and protective efficacy, in comparison to UV and formalin inactivated preparations (329). Intranasal vaccination with γ-FLU was shown to elicit cross-protective T cell responses against heterosubtypic influenza virus challenges, including the highly pathogenic avian H5N1 virus, as well as inducing neutralising antibody responses against homotypic challenges (222, 223). Furthermore, γ-FLU vaccination was reported to behave like a live virus and induce high levels of IFN-I (330, 331). The success of gamma-irradiation as an inactivation method has also been demonstrated for other viruses including rabies (332) and smallpox (333), which highlight the advantage of this inactivation approach for development of effective viral vaccines.

Gamma irradiation has also been used in an attempt to inactivate bacterial pathogens, with gamma irradiated *Rickettsia* being the first reported gamma-irradiated bacterial vaccine capable of inducing protective immunity (334). In addition, gamma irradiated *Listeria monocytogenes* was shown to induce protective $CD4^+$ and $CD8^+$ memory responses comparable to that observed during a live infection, and was also able to provide protection against lethal challenge (335). Similar to that observed with viruses, gamma-irradiation has been reported not to cause any structural damage to bacteria as illustrated with *Brucella abortus*. Furthermore, immunisation with gamma-irradiated *Brucella abortus* was reported to be superior to heat-inactivation at inducing Th1-type immune responses and providing protection in mice (336). The success of gamma-irradiation as an inactivation method has also been applied to other bacterial pathogens such as *Pasteurella tularensis* (337) and *Mycobacterium tuberculosis* (338). Based on these promising observations, gamma irradiation as a technique for pathogen inactivation could be potentially used to develop alternative bacterial vaccines capable of inducing effective immune responses.

1.7.2 Advantages of gamma-irradiation for pathogen inactivation

Gamma-irradiation has increasingly been employed as an inactivation method for pathogens. Many studies have indicated that gamma-irradiation could be a superior inactivation method in comparison to chemical treatment, as it directly damages the genetic material of the microorganism, as opposed to singularly targeting structural proteins. Thus, gamma-irradiation may help maintain structure, integrity and antigenicity of inactivated microbes, which is proposed to facilitate the induction of natural immune responses comparable to the live form of the organism (339). In addition, considering the possible carcinogenic effects of common inactivation chemicals such as β-propiolactone (340, 341), gamma-irradiation may be a much safer alternative. It may also be a simpler method of inactivation as it eliminates the need to remove unwanted chemicals from chemically treated preparations. Based on these principles, the use of gamma-irradiation as an alternative inactivation method deserves further consideration.

Importantly, immunisation with gamma-irradiated rabies virus was shown to be superior to inactivation by β-propiolactone and acetylethyleneimine in protecting mice against rabies infection (332). In addition, a study comparing gamma-irradiated *Toxoplasma gondii* to formaldehyde-treated and native parasites (untreated) also illustrated the superiority of gamma-irradiation as an inactivation technique. Immunisation with gamma-irradiated parasites was shown to induce similar antibody responses to native parasites. More importantly, these mice had extended survival times after tachyzoite challenge relative to mice immunized with formaldehyde-treated parasites, which were unable to induce detectable antibody responses (342). Furthermore, as mentioned above, the effectiveness of gamma-irradiation as an inactivation method compared to other methods has also been clearly demonstrated for influenza and *Brucella Abortus* (329, 336).

Given the advantages reported for gamma-irradiation as an inactivation technique and the utility of this method for development of vaccines for many viral and bacterial pathogens, this technique could be applicable to other notoriously difficult pathogens that are still in need of effective vaccines.

1.8. Research Project

1.8.1. Project rationale

Current licenced pneumococcal conjugate vaccines have limited serotype coverage, are restricted in terms of cost and have lead to replacement of vaccine serotypes with non-vaccine serotypes, causing an increase in disease caused by these types. Therefore, there is a clear demand for an effective pneumococcal vaccine that has broad-spectrum coverage. Previous studies regarding the effectiveness of whole-cell pneumococcal vaccines have utilised chemicals such as ethanol and β-propiolactone as inactivation methods. However, there are safety concerns regarding manufacturing using large volumes of ethanol and there have been reports outlining the possible carcinogenic effects of β-propiolactone. In addition, previous studies evaluating the efficacy of inactivated whole-cell vaccines have employed CT as an adjuvant, which is not suitable for use in humans.

Gamma-irradiation is generally regarded as a superior inactivation method for viral and bacterial pathogens, due to its direct interaction with nucleic acids and the minimal effect on antigenic proteins, in contrast to inactivation using chemicals such as ethanol and β-propiolactone, which can solubilize proteins. Therefore, gamma-irradiation as an inactivation method was investigated in this study to determine whether it could be used to develop an intranasal pneumococcal whole-cell vaccine capable of inducing broad-spectrum immunity without the need to use an adjuvant. Firstly the pneumococcal whole-cell (γ-PN) vaccine was developed. Similar to previously published reports, serotype-specificity was eliminated by employing unencapsulated pneumococcal whole-cells, which are deficient in LytA and express a non-toxic Ply derivative. This allows exposure of all sub-capsular antigens to maximise immunity to multiple pneumococcal targets. After development of the vaccine, γ-PN was then investigated for its immunogenicity and protective efficacy against multiple *S. pneumoniae* serotypes using intranasal vaccination and challenge models. The efficacy of the vaccine was also evaluated when administered with or without mucosal adjuvants. Considering that gamma irradiation has not been previously used as an inactivation strategy for *S. pneumoniae*, this vaccine approach is novel and will provide further insight into the effectiveness of using gamma-irradiation to inactivate Gram-positive bacteria.

The second major part of this study focuses on the problem of co-infection with influenza and *S. pneumoniae*. Current prevention strategies are focused on developing vaccines against individual pathogens in order to reduce hospitalizations and mortality rates associated with co-infection. However, similar to challenges with the current licenced PCV's, licenced influenza vaccines are still not optimal. The inactivated vaccines do not elicit cross protective immunity against newly emerging influenza strains and require annual updating. In addition, the live attenuated vaccines are associated with adverse effects, which restrict certain groups from receiving the vaccine such as the elderly and the immunocompromised. Thus, the lack of optimal vaccines as well as the lethal synergism that occurs between influenza and *S. pneumoniae* illustrate the need for a better vaccination approach against both pathogens.

To my knowledge, there have been no reports of a combination vaccine comprising whole pneumococcal bacterial cells and whole influenza virions to protect against both influenza and *S. pneumoniae* infections. It is well known that the immune mechanisms that dominate against intracellular and extracellular microbes differ significantly. Intracellular pathogens such as influenza most typically promote Th1 immune responses. This is associated with the development of antigen-specific CTLs, which in turn utilise mechanisms such as perforin to destroy viral infected cells. In addition, viral-specific humoral responses are crucial for neutralizing virions to prevent viral attachment to host cells. In contrast, for extracellular microbes such as *S. pneumoniae*, bacterial opsonisation by pneumococcal-specific antibodies in addition to complement is essential to mediate clearance of bacteria by phagocytosis. In addition, the induction of Th17 immune responses promotes neutrophilia, which enhances the process of opsonophagocytosis. In the context of live infection, it is commonly known that viral infections can antagonize the immune responses induced to bacterial pathogens. As previously mentioned, influenza significantly alters the lung physiology and the cytokine milieu, which suppresses bacterial defence mechanisms. This consequently facilitates the persistence of pneumococcal infection within the host (Section 1.3.1). Therefore, based on the difference in immune defence mechanisms directed against whole intracellular and whole extracellular pathogens, it is often assumed that combination of these two types of microbes from a vaccination prospective would not induce optimally immune responses, or could possibly lead to suppression of specific immune mechanisms.

Therefore, considering the previously reported ability of γ-FLU in providing cross protective immunity against lethal influenza strains, and its ability to act like an adjuvant to a co-

administered vaccine, this study also investigated the efficacy of a mucosal combination vaccine comprising gamma-irradiated pneumococcal whole-cells (γ-PN) and gamma-irradiated influenza virions (γ-FLU). The immunogenicity and the ability of the combination vaccine to mediate protection against individual infection with either influenza or *S. pneumoniae* as well as in a model of co-infection was evaluated. If this approach is successful, to my knowledge it will be the first report of a single vaccine containing whole influenza virions and whole pneumococcal cells to protect against both of these pathogens as well as the possibility of co-infection. This would have considerable impact on reduction of morbidity and mortality rates associated with co-infection and would further simplify childhood and elderly vaccination schedules.

1.8.2 Hypotheses and Aims

Hypothesis 1: Gamma-irradiated pneumococcal whole-cells can induce serotype independent protective immunity.

Aim 1.1: To develop an autolysin deficient, pneumolysin mutant (PdT) Rx1 strain Rx1[PdT/ΔLytA].

Aim 1.2: To inactivate the Rx1[PdT/ΔLytA] strain by gamma irradiation and optimise irradiation doses and conditions, designated "γ-PN".

Aim 1.3: To characterise the immune response elicited by the optimised γ-PN preparation and determine the protective efficacy against challenge with distinct *S. pneumoniae* serotypes.

Aim 1.4: To investigate the effect of mucosal adjuvants on the immunogenicity of γ-PN.

Hypothesis 2: Combination of γ-PN and γ-FLU vaccines will generate an effective combination vaccine capable of eliciting protection against individual as well as co-infection with influenza and *S. pneumoniae.*

Aim 2.1: To determine the protective efficacy of the combined gamma-irradiated influenza and pneumococcal vaccines.

Aim 2.2: To characterise the immune responses induced by the combined gamma-irradiated influenza and pneumococcal vaccines.

CHAPTER 2

Materials & Methods

2.1 Bacterial strains and growth conditions

S. pneumoniae strains used in the study have been previously described (343). For challenge studies; D39 (serotype 2) EF3030 (serotype 19F) and P9 (serotype 6A) were grown statically at 37 °C in 5% CO_2 in serum broth (SB) to OD_{600} of 0.18, as previously described (343). Strain Rx1 (capsule-negative strain derived from parent strain D39) and its various mutant derivatives were grown statically in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) at 37°C in 5% CO_2 to OD_{600} of 0.5, as previously described (133).

2.2 Viral stocks

The influenza A viruses, A/PR/8 [(A/Puerto Rico/8/34 (H1N1)] and A/PC (A/Port Chambers/1/73 [H3N2]) were grown in 10-day-old embryonated chicken eggs (HiChick, SA, Australia). Each egg was injected with 0.1 ml normal saline containing 1 hemagglutination unit (HAU) of virus, incubated for 48 hours at 37°C, and then held at 4°C overnight. The amniotic/allantoic fluids were then harvested, pooled, clarified, and stored at −80°C. The titres of these viral stocks were determined by 50% tissue culture infective dose ($TCID_{50}$) assay as previously described (344). The titres were as follows; A/PR8 (~6×10^7 $TCID_{50}$/ml) and A/PC (~3×10^7 $TCID_{50}$/ml).

2.3 Construction of the Rx1 [PdT/ΔLytA] vaccine strain

Genetic manipulation was performed on the 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→Asn, Cys428→Gly and Trp433→Phe), essentially as previously described (345). All PCR primers used are listed in Table 2.1 below. Firstly, an Rx1ΔPly strain was constructed by replacing the Rx1 *ply* open reading frame with an erythromycin resistance (*erm*^{r}) cassette by transformation (345). 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, while 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 (346) using the above three PCR products was then performed to generate the cassette, which was used to transform Rx1, generating Rx1ΔPly. 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 (347) using PdTcloneF and PdTcloneR. The three components of the cassette were then fused and amplified by overlap extension PCR and used to transform Rx1ΔPly, 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, while 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/ΔLytA::*spec*^{r}] construct by transformation with an overlap extension PCR product that fused the *lytA* 5' and 3' flanking regions, thereby deleting the entire *lytA* gene in-frame. The 5' flanking region of *lytA* was obtained by PCR using primers LytAFlankF and LytANullA, while 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/ΔLytA::*spec*^{r}]. The final Rx1[PdT/ΔLytA] construct was validated by PCR, DNA sequencing, Western blotting and a haemolysis assay.

2.4 Generation of the gamma-irradiated Rx1[PdT/ΔLytA] vaccine.

The Rx1[PdT/ΔLytA] vaccine strain was grown in THY as described above, and centrifuged at 4000 × *g* for 10 minutes at 4°C to collect the cells, which were then resuspended in PBS with 10% glycerol at a density of 1×10^{10} CFU/ml. 200 μl volumes were aliquoted into 1.5 ml cryovials and frozen at -80°C. Vials containing the concentrated Rx1[PdT/ΔLytA] 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 gamma-irradiation source and received a dose of 12 kiloGray (kGy). For optimization experiments a range of doses (0.5-25 kGy) were used. The cells were kept frozen on dry ice or room temperature (as a control) during the gamma-irradiation process. The resultant

gamma-irradiated Rx1[PdT/ΔLytA] 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 onto blood agar plates and grown overnight at 37°C in 5% CO_2.

Table 2.1. PCR primers

Primer	5' → 3'
PlyFlankF	GATTGATAATACCAGCACTCAAATTCA
PlyFlankR	ACTTAGCCCGCTTATCAACAGGACTCGCC
PlyEryX	TTGTTCATGTAATCACTCCTTCTAAGTTCCTGGATTGAGTTGCATAAACTC
PlyEryY	CGGGAGGAAATAATTCTATGAGATGCTTGCGACAAAAAGAGGCGAT
J214	GAAGGAGTGATTACATGAACAA
J215	CTCATAGAATTATTTCCTCCCG
PdTcloneF	CGGTAGAGGATTTAAAACAGAGAGGA
PdTcloneR	GAGAGTTGTTCCCCAAATAGAAATCG
PlyRevX	CTTACCTTGATGATTATAGGATAATTC
PlyForY	GAGAGGGTACCGGGCTTGCCTTCGAATGGT
LytAFlankF	TGGGGCTCGGAATTTCACTAAAATC
LytAFlankR	CGTATTCTTCAGTTCCAATGTCTATG
LytA Spec X	TATGTATTCATATATATCCTCCTCTCAATTAAAACAACTCATTTTTTACAAT
LytA Spec Y	AAATAACAGATTGAAGAAGGTATAATAATGGAATGTCTTTCAAATCAGAA
J253	GAGGAGGATATATATGAATACATAC
J254	TTATACCTTCTTCAATCTGTTATTTAAATAGTTTATAGTTA
LytA Null A	TTCTGATTTGAAAGACATTCCATTAATTCTACTCCTTATCAATTAAAACAA
LytA Null B	TTGTTTTAATTGATAAGGAGTAGAATTAATGGAATGTCTTTCAAATCAGAA

2.5 Generation of the gamma-irradiated influenza vaccine

A/PR8 were grown and concentrated as previously described (348). The concentrated stock of A/PR8 (5×10^8 $TCID_{50}$/ml) was maintained frozen on dry ice whilst being inactivated by exposure to gamma-irradiation with a ^{60}Co source (ANSTO), using a dose of 25 kGy, designated 'γ-FLU'. Complete inactivation of the vaccine preparation was confirmed as previously described (348).

2.6 Western blotting

Western blotting was performed essentially as described previously (127). The encapsulated strain D39, the parent Rx1 strain and the vaccine strain Rx1[PdT/ΔLytA] (pre-irradiation) were grown to approximately 2×10^8 CFU/ml and lysates from 20 ml aliquots were analyzed. 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 visualised using the Odyssey imaging system.

2.7 Hemolysis Assay

A hemolysis assay was used to confirm the hemolytic activity of pneumolysin as described previously (349). The parent Rx1 strain and the vaccine strain Rx1[PdT/ΔLytA] (pre-irradiation) were grown to approximately 2×10^8 CFU/ml. 3 ml aliquots of the samples were French pressed at 12,000 psi to obtain lysates for use in the assay.

2.8 Gram staining

20 µl samples of the bacterial vaccine preparations were smeared onto glass slides, heat fixed and then stained with the following reagents in order: crystal violet, Iodine, ethanol 95% and safranin. All reagents were placed on the slides for 30 seconds and rinsed off with distilled water between each stain. After the slides were dried, each Gram-stained smear was evaluated under oil immersion (×100 objective) using a light microscope.

2.9 Scanning electron microscopy

~3.5×10^8 CFU of the non-irradiated vaccine strain Rx1[PdT/ΔLytA] (Control) or irradiated vaccine samples (12, 18 or 24 kGy) were filtered through a Whatman Nucleopore Track-Etch

membrane and then fixed with EM fixative (4% paraformaldehyde/1.25% glutaraldehyde in PBS, + 4% sucrose at pH 7.2) for 30 minutes at room temperature (RT). The membrane was washed in buffer (PBS + 4% sucrose) and then immersed in 2% Osmium tetroxide (OsO4) diluted in water for 30 minutes at RT. The fixed bacteria were then dehydrated with increasing concentrations of ethanol (70%, 90%) for 10 minutes each at RT followed by 100% ethanol for 3 × 10 minutes. The membrane was then immersed in a solution containing hexamethyldislazane (HMDS) and ethanol (100%) (1:1 ratio) for 10 minutes and then subjected to a final incubation with 100% HMDS. Membranes were left to air dry. Following coating by gold sputter, the bacteria were observed using a Philips XL30 scanning electron microscope.

2.10 Focus forming inhibition assay

Focus forming inhibition assay was performed as described previously (70) using sera from mice immunized with γ-FLU, γ-PN or γ-PN + γ-FLU. Sera from PBS vaccinated mice served as the control. Sera were pooled from 10 mice within each group to perform serial dilutions. Images were acquired using a Nikon TiE inverted fluorescence microscope and analyzed using NIS elements software (Tokyo, Japan).

2.11 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 (7^{th} Edition [2004] and 8^{th} Edition [2013]) and the South Australian Animal Welfare Act 1985. Protocols were approved by the Animal Ethics Committee at The University of Adelaide (Approval numbers S-2010-001 and S-2013-053).

2.12 Vaccination

Wild-type C57BL/6 mice (5-6 week old) were supplied by Laboratory Animal Services at The University of Adelaide, South Australia. For intranasal vaccinations, mice were anaesthetized intraperitoneally (IP) with pentobarbital sodium (Nembutal; Ilium; 66 mg/g body weight) and then administered the relevant vaccine preparation: γ-PN (~1×10^8 CFU-equivalent/dose) or γ-FLU (~1×10^6 $TCID_{50}$-equivalent/dose) in 30 μl intranasally (IN). For co-vaccination (γ-PN + γ-FLU), the appropriate doses of each of the 2 vaccines were combined in a final volume of 30 μl and administered in the same manner. Control mice

received PBS. The mice were immunized intranasally twice at two-week intervals and sera were collected from all experimental animals by submandibular bleeding one week after the last immunization. For IP vaccinations, mice received γ-PN (~1 × 10^8 CFU-equivalent/dose) in 200 μl volume in PBS.

2.13 Adjuvants

For all immunizations involving adjuvants, the appropriate doses of cholera toxin (1 μg) (Sigma) or Mannosylated chitosan (stock = ~2 mg/ml) (kind gift from Dr Billy Hargis, Poultry Health Laboratory, University of Arkansas, USA) (dose specified in figure legends) were administered intranasally with the appropriate vaccination dose of γ-PN (~1 × 10^8 CFU-equivalent/dose) in a total volume of 30 μl.

2.14 Infection models

Intranasal sepsis model: Two weeks after the second IN vaccination, vaccinated and control (PBS-treated) mice were anaesthetized and challenged with strains D39 (1 × 10^6 CFU) or P9 (5 × 10^6 CFU) in 30 μl. Mice were then monitored for up to 21 days or until they were moribund (i.e. hunched posture, inactivity, ruffled fur and labored breathing).

Intranasal colonization-induced pneumonia model: Following immunization procedures, un-anaesthetized C57BL/6 mice were infected with *S. pneumoniae* strain EF3030 (serotype 19F) by gently restraining the mice and applying 10 μl containing 1 × 10^7 CFU to the nostrils to allow for bacterial colonization. Four days later, the mice were anaesthetized and given 30ul PBS IN. Seven days later, the mice were euthanized and the nasal wash, nasopharyngeal tissue and lungs were harvested, homogenized where necessary and plated for CFU recovery, as described previously (350), in 1ml. Total CFU in the nasopharynx includes the CFU recovery from the nasal wash and nasal tissue combined.

Intranasal colonization model: Mice were colonized with EF3030 as described above and 7 days later, nasopharyngeal bacterial loads were analyzed as described above.

Intranasal influenza challenge model: Following immunization, mice were challenged IN with either A/PR8 (~200 $TCID_{50}$) or A/PC (~1 × 10^4 $TCID_{50}$) in 30 μl and the weight of the mice was recorded daily for 21 days. Percentage weight loss was calculated from original weight before challenge. Mice were sacrificed at 20% weight loss as per ethical guidelines.

Co-infection model: Following immunization, mice were colonized with EF3030 as described above. 4 days later, mice were anaesthetized and infected IN with a subclinical dose of

A/PR8 (10 $TCID_{50}$ in 30 μl) or PBS (control). Mice infected with EF3030 or A/PR8 alone served as controls. All mice were monitored for up to 21 days post co-infection.

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

Two weeks after mice received the second dose of γ-PN vaccine as described above, they were treated with neutralising monoclonal antibodies (all from BioxCell) to IL-17A or IFN-γ (351). Mice were administered 3 IP doses (200 μg in 200 μl PBS per dose) of either anti-IL-17A (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 hours before the challenge), day 0 (6 hours post challenge) and day 2 (48 hours 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.

2.16 CD4 depletion *in vivo*

Mice were treated with a depleting monoclonal antibody (BioxCell) to CD4 prior to and during challenge in the colonization-induced pneumonia model. Mice were given 4 doses intraperitoneally (150 μg in 200 μl PBS/dose) of either anti-CD4 (GK1.5) or anti-rat IgG2b (LTF-2; isotype control) at day -2, -1, 1 and 4 (6 hours before intranasal administration of PBS). Successful CD4 cell depletion in the lung and spleen were confirmed using flow cytometry.

2.17 Measurement of antibody responses

Sera collected from immunized mice were assayed by ELISA to determine *S. pneumoniae*-specific and A/PR8-specific antibody responses, as described previously (343, 348). Alkaline phosphatase (AP) conjugated goat anti-mouse IgA (Zymed), horse radish peroxidase (HRP) conjugated goat anti-mouse IgG (Thermo Scientific) and alkaline phosphatase (AP) conjugated goat anti-mouse IgG (Zymed) detection antibodies were used in the study. For *S. pneumoniae*-specific antibody analysis, the whole-cell vaccine strain (intact WC) was prepared as follows. Rx1[PdT/ΔLytA] was grown statically in THY at 37°C in 5% CO_2 to OD_{600} of 0.5. The preparation was washed twice with PBS and resuspended in a 1:1 ratio in PBS and 100% glycerol to a concentration of 5×10^{10} CFU/ml. The stock was maintained at -20°C. ELISA plates were coated with 50 μl per well of a 10^8 CFU/ml bacterial suspension

obtained by diluting the stored stock in bicarbonate coating buffer. The WC lysate was prepared as follows. The vaccine strain Rx1[PdT/ΔLytA (pre-irradiation) were grown (as above) to approximately 2×10^8 CFU/ml. A 3 ml aliquot of the sample was French pressed at 12,000 psi to obtain the "lysate" and 100% glycerol was added at 1:1 ratio. The stock was maintained at -20°C. ELISA plates were coated with 100 μl of WC lysate diluted in bicarbonate coating buffer (5 μg/ml) from the stored stock. Similarly for detection of antibodies specific for pneumococcal proteins, ELISA plates were coated with 100 μl of purified proteins (Ply, CbpA, PspA)(118, 119) diluted in bicarbonate coating buffer (5 μg/ml). 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 standard deviations (SD) to the mean (i.e. mean + 3SD) of the OD values of samples from the negative control PBS treated mice.

Sera were also collected an analysed for IFN-α levels using the VeriKine™ Mouse Interferon Alpha ELISA kit, as per manufacturers' instructions.

2.18 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 (85) with a few modifications**.** To obtain cell suspensions, splenocytes were passed through a 70 μm 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% β-mercaptoethanol). Erythrocytes were then lysed using red blood cell lysis buffer. Splenocytes were plated into 96 well tissue culture plates at a concentration of ~1.8×10^6 cells in 200 μl. Splenocytes were stimulated with 50 μl of complete RPMI containing either γ-PN (1×10^6 CFU/ml equivalents), γ-FLU (2×10^7 $TCID_{50}$/ml equivalents) or MalX antigen (10 μg/ml). Complete RPMI 1640 media alone served as a control. 72 hours later, cells were collected following centrifugation and used for intracellular cytokine staining. Culture supernatants were analyzed for levels of IL-17A (R&D systems) and IFN-γ (BD) using ELISA kits, as per manufacturers' instructions.

2.19 Lung digestion to retrieve lymphocytes for flow cytometric analysis

Lungs were collected from immunized mice at 24 and 48 hours post challenge. The lungs were perfused with cold PBS and subsequently dissected and placed in 1 ml digestion medium (DMEM containing sodium pyruvate (Thermo Fisher Scientific) supplemented with 1% HEPES , 5% FCS, 1% penicillin/gentamicin, 2.5 mM $CaCl_2$, collagenase D (Roche) (1 mg/ml) and DNase (40 U/ml; Roche)). Lungs were then finely macerated with surgical scissors and incubated at 37 °C for 1 hour (with mixing every 20 minutes). Samples were then processed to obtain single cell suspensions as described above and subjected to intracellular cytokine staining.

2.20 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 (352). For stains involving Foxp3 detection, cells were permeabilized using the Foxp3/transcription factor staining buffer set (eBioscience), while for all other stains the Cytofix/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-γδTCR (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 analyzes using LIVE/DEAD fixable near-infrared dye (Molecular Probes). Cells were acquired using an LSRII flow cytometer (BD) and data was analyzed using FlowJo software (TreeStar).

2.21 In vivo cytotoxic T cell assay

Mice were immunized IV with live A/PR8, γ-FLU or given PBS (dose specified in Figure 5.4). 1 week post immunization, target splenocytes harvested from $CD45.1^+$ mice were divided into 2 populations, either labelled with Carboxyfluorescein succinimidyl ester

(CFSE) and pulsed with nucleoprotein peptide (NPP), or left unpulsed and unlabelled. Cell populations were mixed in equal proportions and ~1 × 10^7 cells were adoptively transferred IV into immunized or PBS immunized recipient mice ($CD45.2^+$). 18 hours post injection of target splenocytes, the spleens were harvested and analyzed for the percentage of labelled target cells ($CFSE^+$, 'pulsed') by flow cytometry. Cytotoxic T cell killing was calculated as ((percentage of pulsed cells remaining – 50%) / 50%). 50% represents the percentage of pulsed cells remaining in naïve mice.

2.22 Statistics

Data were analyzed using a Fisher's exact test, unpaired Student's *t*-test, Mann-Whitney *U*-test, or a One-way ANOVA with a Tukey multiple comparisons test as appropriate, as outlined in the text. Results with *P* values less than 0.05 were considered statistically significant. Statistical analyzes were performed using Graph Pad Prism.

CHAPTER 3

The efficacy of the γ-PN vaccine

3.1 Introduction

Since the introduction of PCV's into routine childhood immunisation schedules, serotypes not included in vaccine formulations have replaced pneumococcal strains normally harboured within the nasopharynx. Consequently, this has resulted in increased incidence of invasive pneumococcal disease caused by these non-vaccine serotypes (98). In addition, the increased prevalence of these strains coupled with continued antibiotic use has also corresponded to an increase in antibiotic resistance (107-109). Therefore, in light of existing scientific challenges associated with currently licenced pneumococcal vaccines, there is a clear need to develop serotype-independent pneumococcal vaccines. Inactivated whole un-encapsulated bacterial cells as opposed to selected antigens have recently been proposed as a serotype-independent vaccine candidate (353). Unlike live-attenuated vaccines, complete inactivation of whole-cells eliminates the concerns regarding possible reversion to full virulence by live attenuated vaccine strains. Moreover, removal of the CPS exposes a plethora of sub-capsular antigens capable of inducing immunity. In particular, serotype-independent protection has been related to the induction of protective Th17 cell-derived IL-17 responses. The importance of IL-17 responses in vaccine efficacy is becoming more apparent and has also been demonstrated for other pneumococcal vaccines (86, 137, 354, 355). However, common approaches for inactivation of whole-cell-based vaccines include heat or ethanol treatment; both of these methods have been reported to affect membrane integrity of bacterial cells and subsequently influence vaccine immunogenicity (335, 356). β-propiolactone has also been employed as a method of inactivation (136, 139), but reports of carcinogenic effects question its safety for human use (340, 341). Overall, safer alternative inactivation methods are required to maintain pneumococcal antigens in their native form, thereby enhancing vaccine immunogenicity.

The possibility of using gamma-irradiation to inactivate pathogens for vaccine purposes has been investigated (224). Gamma-irradiation directly inactivates pathogens by targeting the genetic material with limited effects on proteins. Subsequently, it has been proposed to preserve antigenic structures of proteins, which can behave in similar manners to that of live microbe (319, 325, 329). This inactivation approach has been adopted for various viral and bacterial vaccines and have shown remarkable efficacy (222, 333, 335, 336). Studies have also clearly demonstrated the advantageous effects of gamma-irradiation in comparison to other inactivation methods such as formaldehyde, UV, β-propiolactone and acetyl-ethyleneimine (329, 332).

In the present study, gamma-irradiation was utilised to inactivate an un-encapsulated whole-cell *S. pneumoniae* strain Rx1. The *lytA* gene was deleted and the *ply* gene was replaced with an allele encoding a non-toxic pneumolysoid (PdT). LytA is responsible for cell autolysis during growth, while Ply is a pore forming toxin that plays many roles in *S. pneumoniae* pathogenesis including a major role in immune evasion. The immunogenicity and protective efficacy of the gamma-irradiated pneumococcal whole-cell vaccine, designated "γ-PN" was investigated using intranasal vaccination and challenge models.

3.2 Results

3.2.1 Characterization and optimization of the γ-PN vaccine

The γ-PN vaccine (Rx1[PdT/ΔLytA]) 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→Asn, Cys428→Gly and Trp433→Phe) that abrogate both the complement activation and cytotoxic properties of the toxin, without affecting immunogenicity (347, 357) (See Methods 2.3). 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 (Fig. 3.1A). Anti-Ply and anti-LytA antibodies detected both LytA and native Ply in the encapsulated strain (D39), the parent strain (Rx1) and the respective purified control proteins. As expected, no LytA related bands were detected in lysate of pre-irradiated vaccine strain Rx1[PdT/ΔLytA]. In contrast, bands related to Ply were still detectable.

To determine the hemolytic activity of the final vaccine strain Rx1[PdT/ΔLytA] (pre-irradiation) which produces a non-toxic derivative of Ply, a hemolysis assay was performed (Figure 3.1B). The wild type strain Rx1, which contains a functional *ply* gene displayed hemolytic activity. As expected, complete absence of hemolytic activity was confirmed in the vaccine strain Rx1[PdT/ΔLytA] and this was similar to that observed with the strain containing the antibiotic resistance gene ermR in place of the *ply* gene, Rx1[erm^R/ΔLytA]. 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 (data not shown).

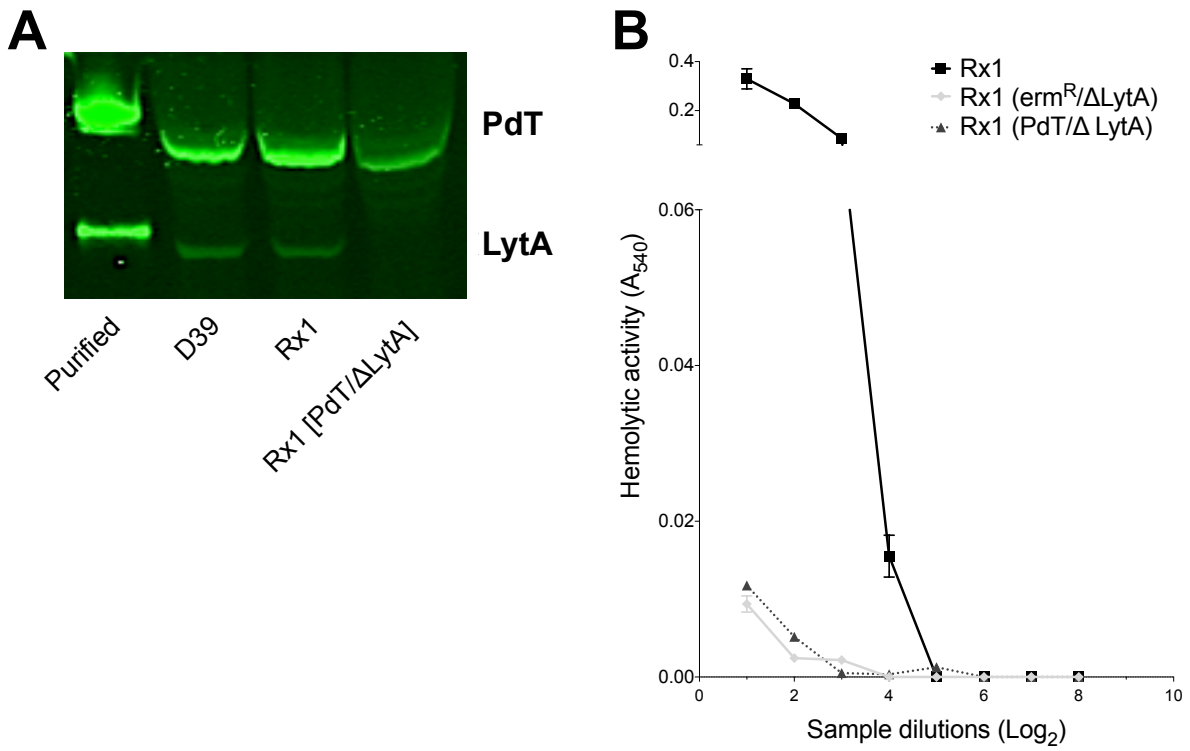


Figure 3.1: Expression of PdT and LytA and hemolytic activity of the vaccine strain Rx1[PdT/ΔLytA]. (A) Expression of Ply/PdT (53 kDa) and LytA (36 kDa) was determined by Western blotting in the *S. pneumoniae* strain D39; Rx1 and the Rx1[PdT/ΔLytA] vaccine strain (pre-irradiation). Recombinant purified Ply and LytA (5 ng each) served as controls. The 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 hemolysis assay. Hemolytic activity represented as mean ± SEM (n = 3).

Considering that gamma-irradiation has not been applied previously for inactivation of *S. pneumoniae* bacterial cells, samples of the vaccine preparation were exposed at the concentration of ~2×10^{10} CFU/ml to different doses of gamma-irradiation (0.5- 24 kGy) and conditions (dry ice (DI) versus room temperature (RT) as a control) to determine the dose required to completely inactivate the vaccine preparation. Samples were then plated onto blood agar plates for enumeration of CFU. As shown in Figure 3.2, samples that were irradiated on DI required higher doses of gamma-irradiation to completely inactivate preparations in comparison to those irradiated at RT. 4 kGy and 12 kGy were the minimum doses required for complete inactivation of preparations irradiated at RT and DI, respectively.

It has been reported that temperature conditions can affect the inactivation process by gamma-irradiation. Higher temperatures have been shown to enhance the indirect effect of gamma-irradiation thereby causing the generation of free radicals (358). This consequently may affect protein structures. Therefore, to determine the impact of increasing doses of gamma-irradiation and temperature conditions on the cell wall integrity of pneumococcal whole-cells, samples of the vaccine strain irradiated at 12, 18 and 24 kGy either at RT or on DI were subjected to Gram staining. *S. pneumoniae* is a Gram-positive bacterium as it contains a thick layer of peptidoglycan in its cell wall, therefore pneumococcal cells are expected to stain purple as it retains the crystal violet dye. As observed in Figure 3.3A, vaccine samples irradiated at RT (12, 18 or 24 kGy) show a significant disruption of bacterial cells. In addition, remaining intact bacterial cells in these samples were stained pink, indicating the loss of retention of the crystal violet dye. Interestingly, pink staining was also observed for the non-irradiated control sample at RT, which indicate some perturbation of pneumococcal cells but to a lower extent compared to the irradiated samples. For DI preparations, pneumococcal whole-cells that were exposed to 12 kGy appeared structurally intact (Figure 3.3B). The majority of bacterial cells in this sample were stained purple with the presence of some pink stained bacterial cells similar to that observed with the DI control (non-gamma-irradiated pneumococci). In addition, bacterial cells subjected to higher gamma-irradiation doses (18 kGy and 24 kGy) on DI also appeared structurally intact, but there was a higher proportion of bacterial cells stained pink relative to the DI control and 12 kGy preparations (Figure 3.3B). Overall, these data suggest that gamma-irradiation on DI is associated with reduced structural damage to pneumococcal cells compared to irradiation at RT.

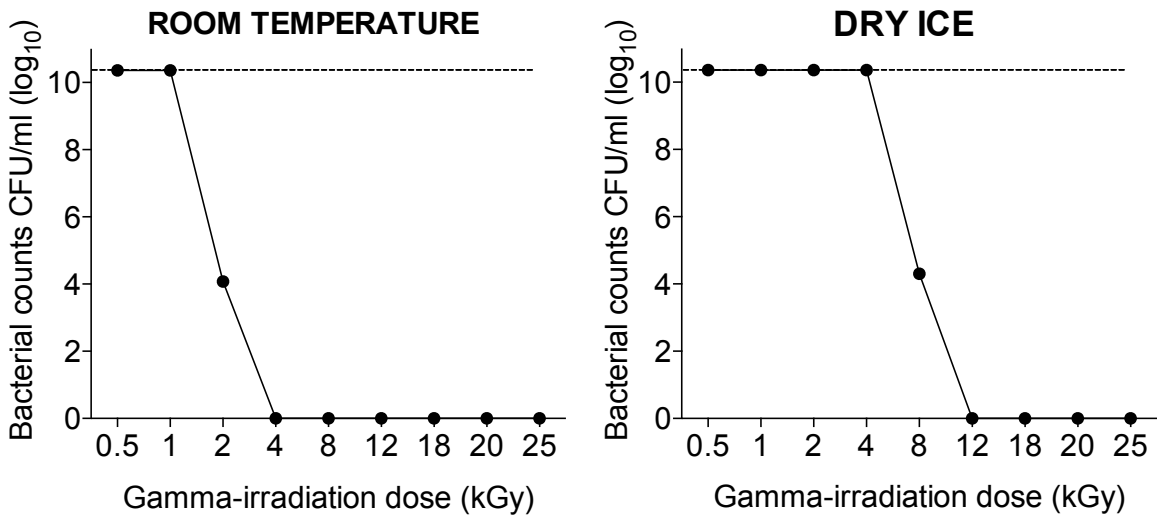


Figure 3.2: Sterility testing of the gamma-irradiated vaccine strain Rx1[PdT/ΔLytA]. Samples of the vaccine strain Rx1[PdT/ΔLytA] were gamma-irradiated at different doses (0.5-24 kGy) and treated either on dry ice (DI) or room temperature (RT). Post gamma-irradiation, the numbers of CFU were determined in each sample. Graphs represent concentration (CFU/ml) of viable bacteria. Baseline represents no detectable bacterial colonies (complete inactivation).

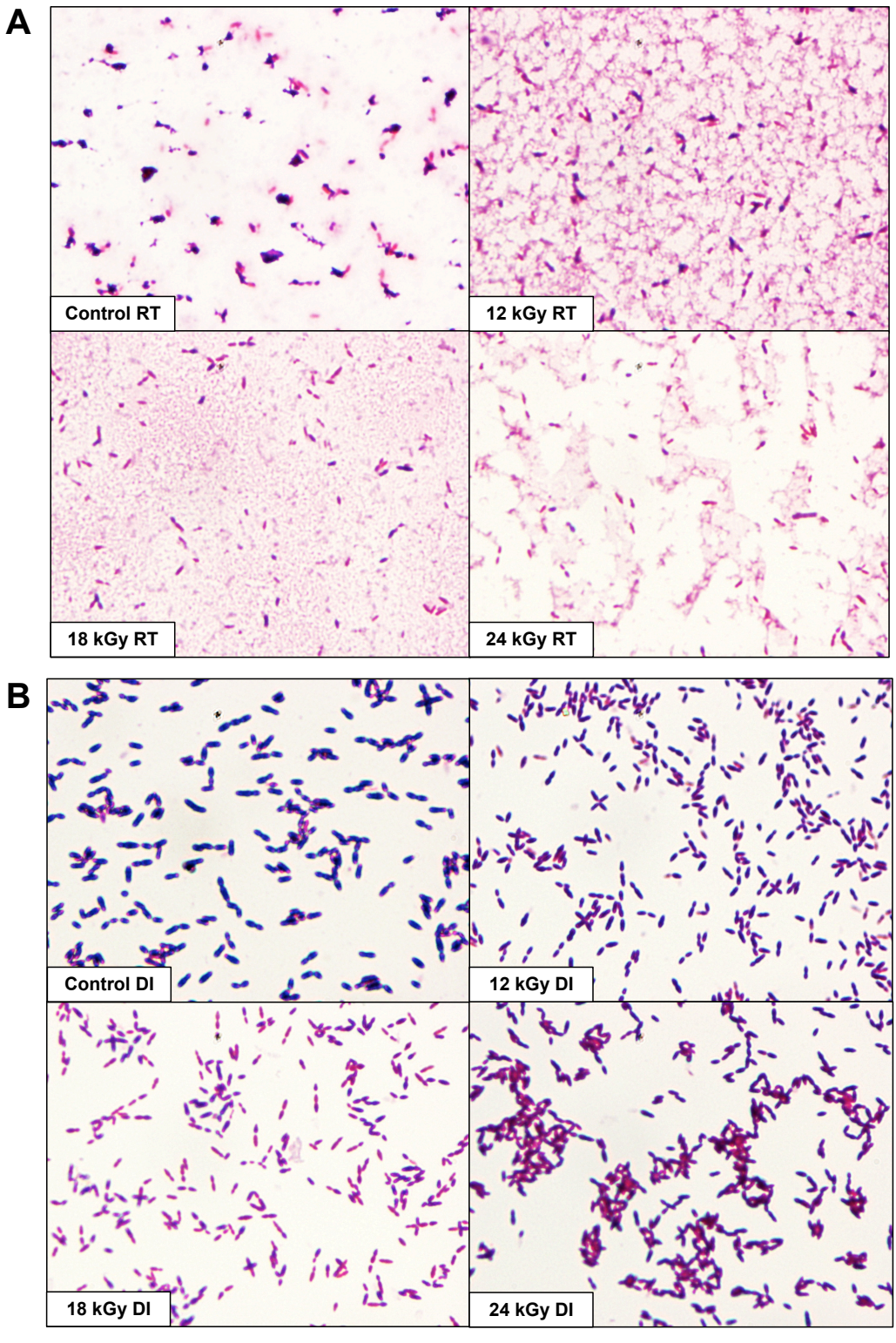


Figure 3.3: High doses of gamma-irradiation may affect the integrity of the cell membrane. Samples of vaccine preparations exposed to various doses of gamma-irradiation (12, 18 or 24 kGy) at either (A) room temperature (RT) or (B) dry ice (DI), were subjected to Gram staining. Non-irradiated samples at the relevant temperature conditions were used as a control.

Samples irradiated on DI at 12, 18 & 24 kGy were also analyzed using scanning electron microscopy (Section 2.9). Gamma-irradiation did not have a significant effect on the structure of the bacterial cells as the morphology was similar to that observed with the non-irradiated control (Figure 3.4). The morphology was also comparable among the varying doses of gamma-irradiation analyzed. Thus, in the context of Gram staining, these results confirm that the gross overall structure of pneumococcal cells can still be maintained after gamma-irradiation on DI with doses of up to 24 kGy.

Next, the effect of irradiation dose on the immunogenicity of inactivated preparations was investigated, focusing on materials irradiated on DI. Mice were vaccinated intraperitoneally with 2 doses of vaccine preparations irradiated at 12, 18 or 24 kGy. Two weeks post the second immunisation dose, serum samples were collected and analyzed by ELISA for IgG-specific antibodies against the whole-cell lysate and purified protein antigens (Ply, CbpA, PspA). All vaccine preparations were capable of inducing antibody responses against Ply, CbpA, PspA and the WC lysate (Figure 3.5A&B). At the dilution of 1/800, the 12 kGy vaccine preparation induced significantly higher antibody levels in comparison to the PBS control against the WC lysate, Ply and CbpA. In addition, the 12 kGy vaccine preparation appeared to induce higher antibody levels compared to the 18 and 24 kGy preparations. Importantly, statistically significant increases were observed for antibody responses induced by the 12 kGy preparation relative to the 24 kGy preparation against the WC and Ply. These data suggest that increasing doses of gamma-irradiation may reduce the immunogenicity of vaccine preparations.

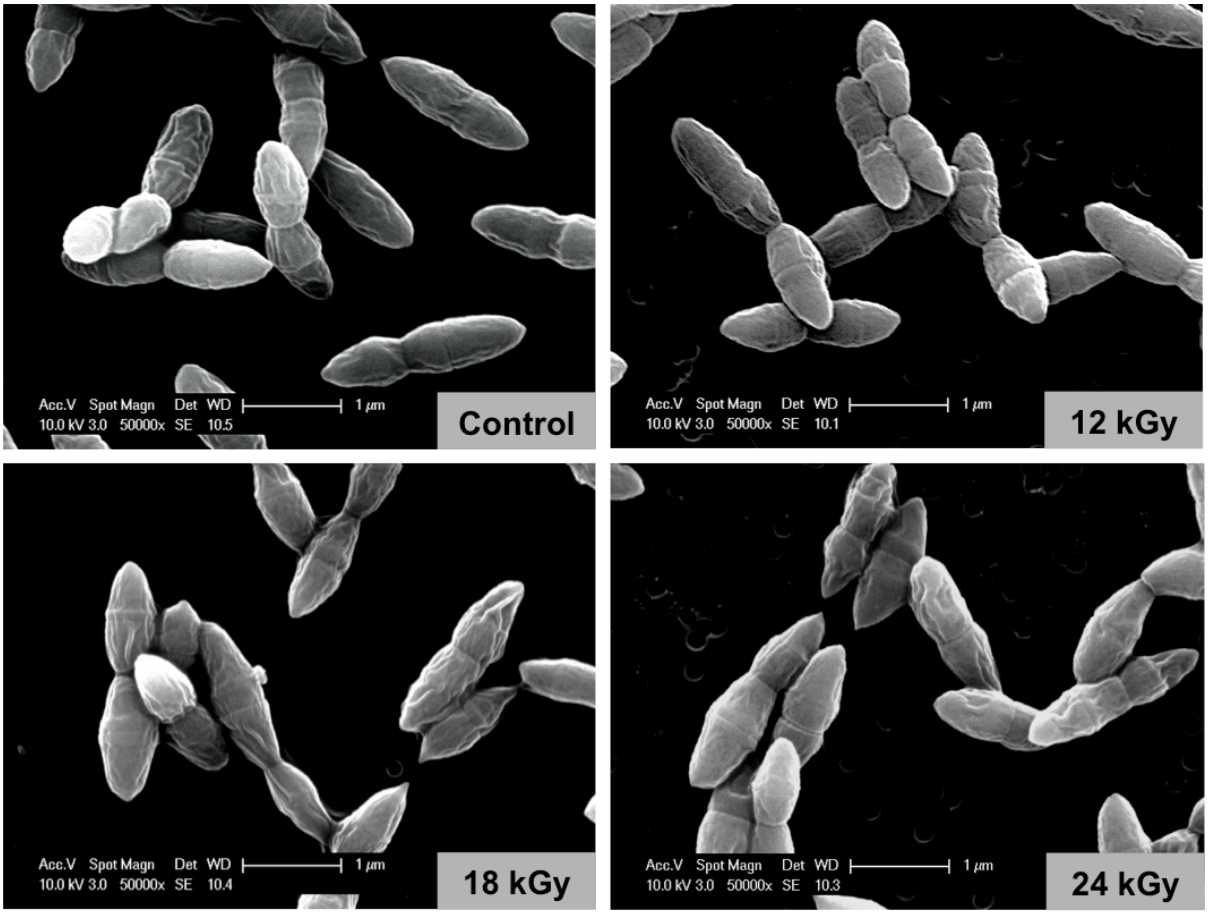


Figure 3.4: Gamma-irradiation does not affect the morphology of *S. pneumoniae*. Samples of vaccine preparations exposed to various doses of gamma-irradiation (12, 18 or 24 kGy) on DI were prepared and analyzed by scanning electron microscopy. Images taken at 500000× magnification show the morphology of pneumococcal cells (strain Rx1[PdT/ΔLytA]) after exposure to increasing doses of gamma-irradiation (12, 18 or 24 kGy). Un-irradiated pneumococcal cells (strain Rx1[PdT/ΔLytA]) served as the control.

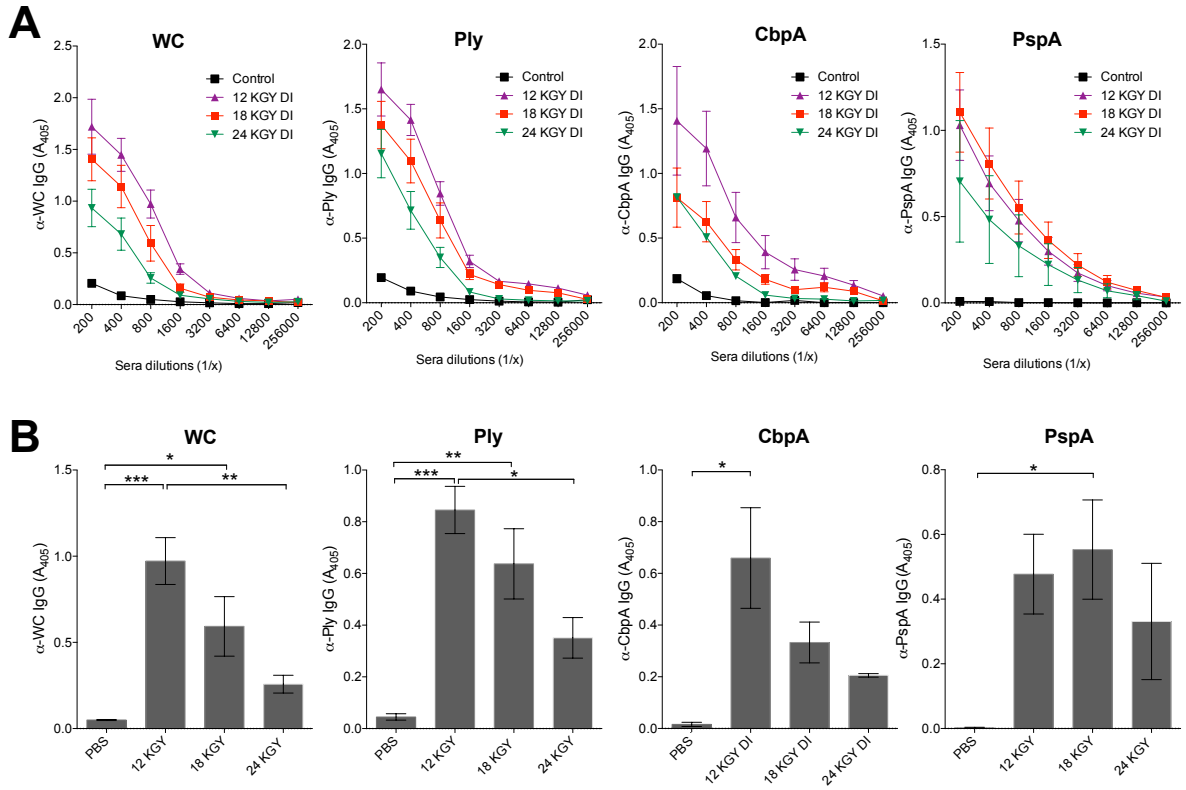


Figure 3.5: Higher doses of gamma-irradiation appear to affect the immunogenicity of vaccine preparations. Mice were immunized IP with 2 doses of γ-PN (12, 18 or 24 kGy) in a volume of 200 µl and mice treated with PBS were used as a control (Section 2.12). 2 weeks after the second immunisation, sera was collected and assayed for IgG-specific antibodies against the whole-cell lysate (WC) or purified proteins (Ply, CbpA and PspA) by ELISA (A) Represents absorbance readings for serial dilutions of sera for each of the vaccine groups. (B) Relative antibody levels at the dilution of 1/800. Antibody levels are represented are mean ± SEM (n = 5). Data were analyzed using a One-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.2.2 Immunisation with γ-PN provides serotype-independent protection

Considering the effectiveness of 12 kGy DI reported in terms of immunogenicity and membrane integrity, all further experimental approaches were carried out with the 12 kGy DI preparation (designated "γ-PN"). Given that the γ-PN vaccine is derived from the un-encapsulated strain Rx1, the vaccine was initially investigated for its ability to provide protection against intranasal challenge by the ancestral encapsulated (type 2) strain D39. The data show that intranasal vaccination of mice with γ-PN resulted in a statistically significant increase in survival rate (P = 0.0201) and survival time (P = 0.0226) compared to mock-immunized control mice following lethal challenge with *S. pneumoniae* D39 (Figure 3.6A&B). To investigate whether the vaccine can provide serotype-independent protection, γ-PN immunized mice were challenged with the unrelated *S. pneumoniae* strain P9 (serotype 6A). Similarly, intranasal vaccination with γ-PN afforded significant protection against P9 challenge (P = 0.0431, survival rate, P = 0.0078 survival time) (Figure 3.6A&B), indicating that the γ-PN vaccine is capable of providing both serotype-dependent and -independent protection against lethal pneumococcal challenge.

In addition, the γ-PN vaccine was also investigated for its ability to elicit protection against local infection in the lung. Thus, immunized mice were challenged under anaesthesia with EF3030 (serotype 19F), which causes focal pneumonia but not lethal sepsis (209, 359) and bacterial counts in the lungs and nasopharynx were determined 4 days post challenge. The data show that vaccination with γ-PN reduced bacterial counts in the lungs (P = 0.0397) when compared to control mice (Figure 3.7A). Although median CFU in the nasopharynx was also slightly lower in γ-PN vaccinated mice, this did not reach statistical significance (Figure 3.7A). To further explore whether effects on colonization might contribute to vaccine efficacy, a colonization model was utilised. Vaccinated and control mice were inoculated intranasally with a lower dose of *S. pneumoniae* EF3030 without anaesthesia and bacterial counts were determined in the nasopharynx at day 7 post inoculation. The 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 3.7B).

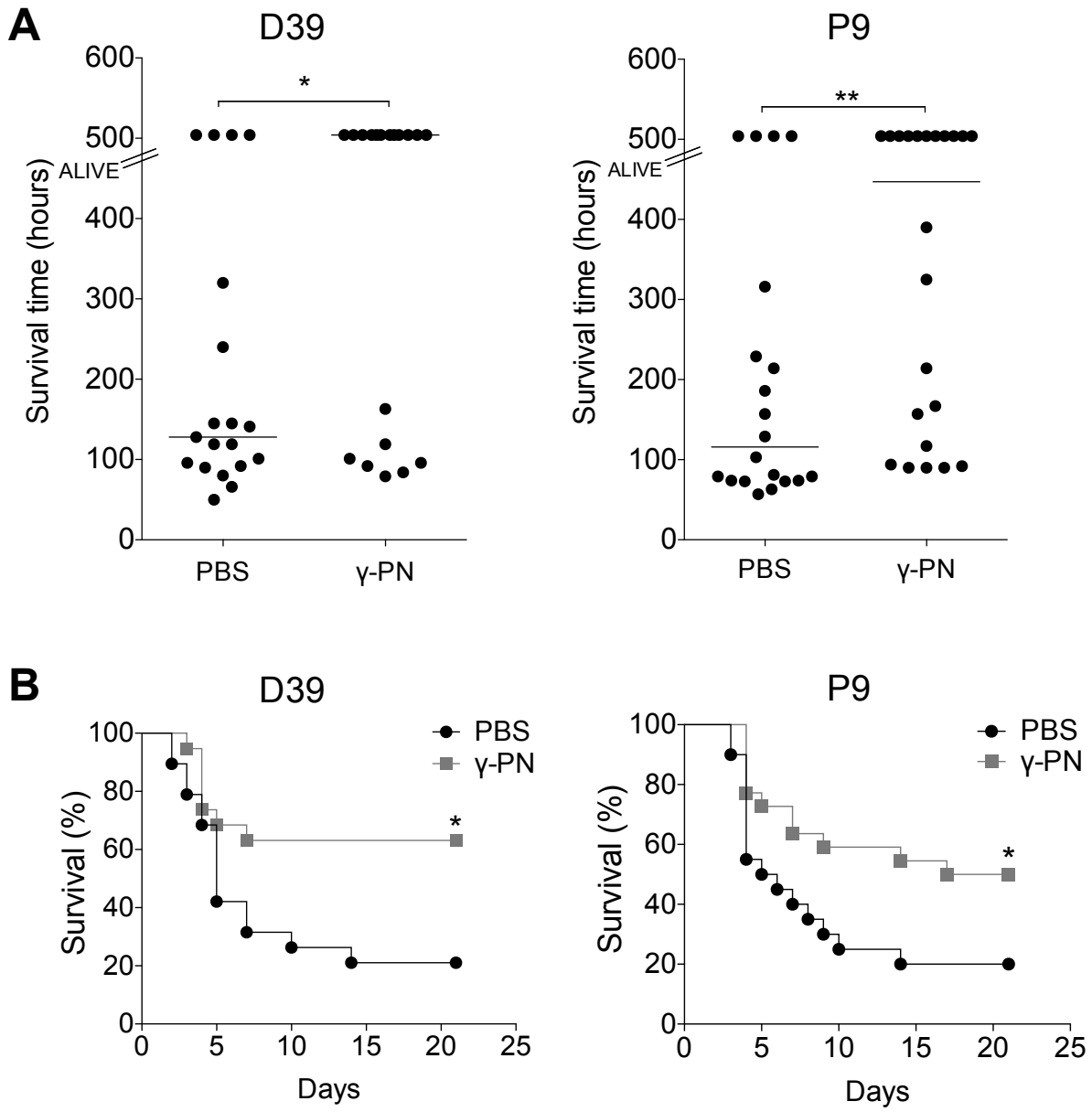


Figure 3.6: Protection against sepsis elicited by immunisation with γ-PN. Mice (n = 20; data pooled from 2 independent experiments of n = 10) were immunized IN with 2 doses of γ-PN or given PBS (Section 2.12). 2 weeks after the second immunisation, mice were challenged IN under anaesthesia with *S. pneumoniae* D39 or P9 (Section 2.14). (A) Survival time and (B) overall survival rate was determined at 21 days. Data were analyzed using a Mann-Whitney *U*-test (survival time) or a Fisher exact test (survival rate) (*, $P < 0.05$; **, $P < 0.01$).

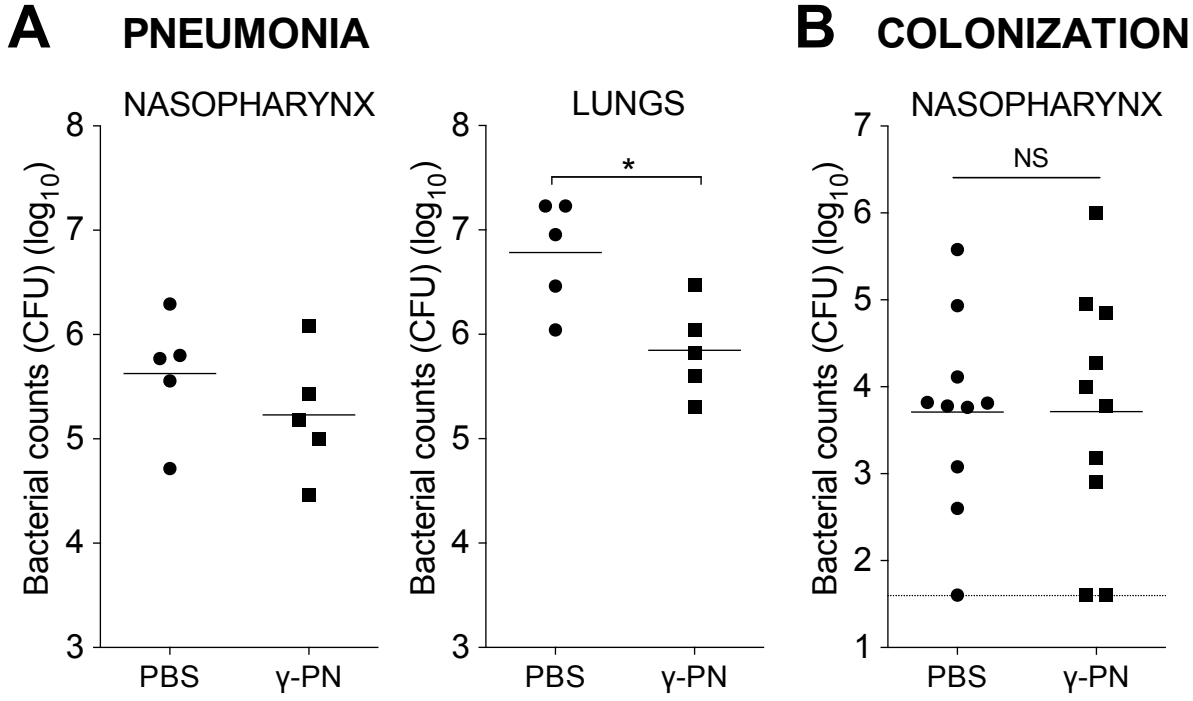


Figure 3.7: Protection against focal pneumonia elicited by immunisation with γ-PN. Mice were immunized IN with 2 doses of γ-PN or given PBS (Section 2.12). 2 weeks after the second immunisation, mice were challenged IN with (pneumonia model) or without (colonization model) anaesthesia with *S. pneumoniae* EF3030 (Section 2.14). (A) For the pneumonia model, bacterial counts in the lungs and nasopharynx 4 days following challenge are shown (n = 5). (B) For the colonization model, bacterial counts in the nasopharynx at 7 days post inoculation are shown (n = 10). Dotted line represents detection limit. Differences in bacterial load were analyzed using the Mann-Whitney *U*-test (*, $P < 0.05$).

3.2.3 γ-PN efficacy is dependent on B cell responses

To elucidate the underlying mechanisms of γ-PN-mediated protection, the role of B cell responses in acquired immunity to *S. pneumoniae* were examined. Induction of antibody-initiated complement-dependent killing represents the hosts' major defence mechanism against pneumococcal challenge (1, 89), and current approaches for pneumococcal vaccines rely principally on the efficacy of B cell responses (353). Thus, to evaluate the ability of γ-PN to induce antigen-specific antibody responses, IgG responses to pneumococcal antigens (Ply, CbpA and PspA) as well as against the intact WC strain were analyzed. The WC intact strain was used instead of the WC lysate (previously used with I.P immunization) as antibody responses could not be detected against the WC lysate in sera from intranasally immunized mice (antibody levels were below detection limit). As shown in Figure 3.8A, significant antibody levels (IgG and IgA) were detected against the intact WC in sera collected from mice vaccinated with γ-PN in comparison to the PBS control. While there were no detectable antibodies against Ply or CbpA (data not shown), significant levels of PspA-specific IgA and IgG were detected (Figure 3.8A).

Next, to determine whether the vaccine's efficacy is dependent on B cell responses, μMT 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 3.8B, γ-PN-vaccinated WT mice show significant protection against lethal D39 challenge compared to PBS treated controls, as indicated by a significantly greater survival rate (P = 0.0059). In contrast, γ-PN-immunized B cell-deficient μMT mice did not show protection when compared to PBS treated μMT control mice (Figure 3.8B). This suggests that the protective efficacy of the γ-PN vaccine against sepsis is reliant on B cell responses.

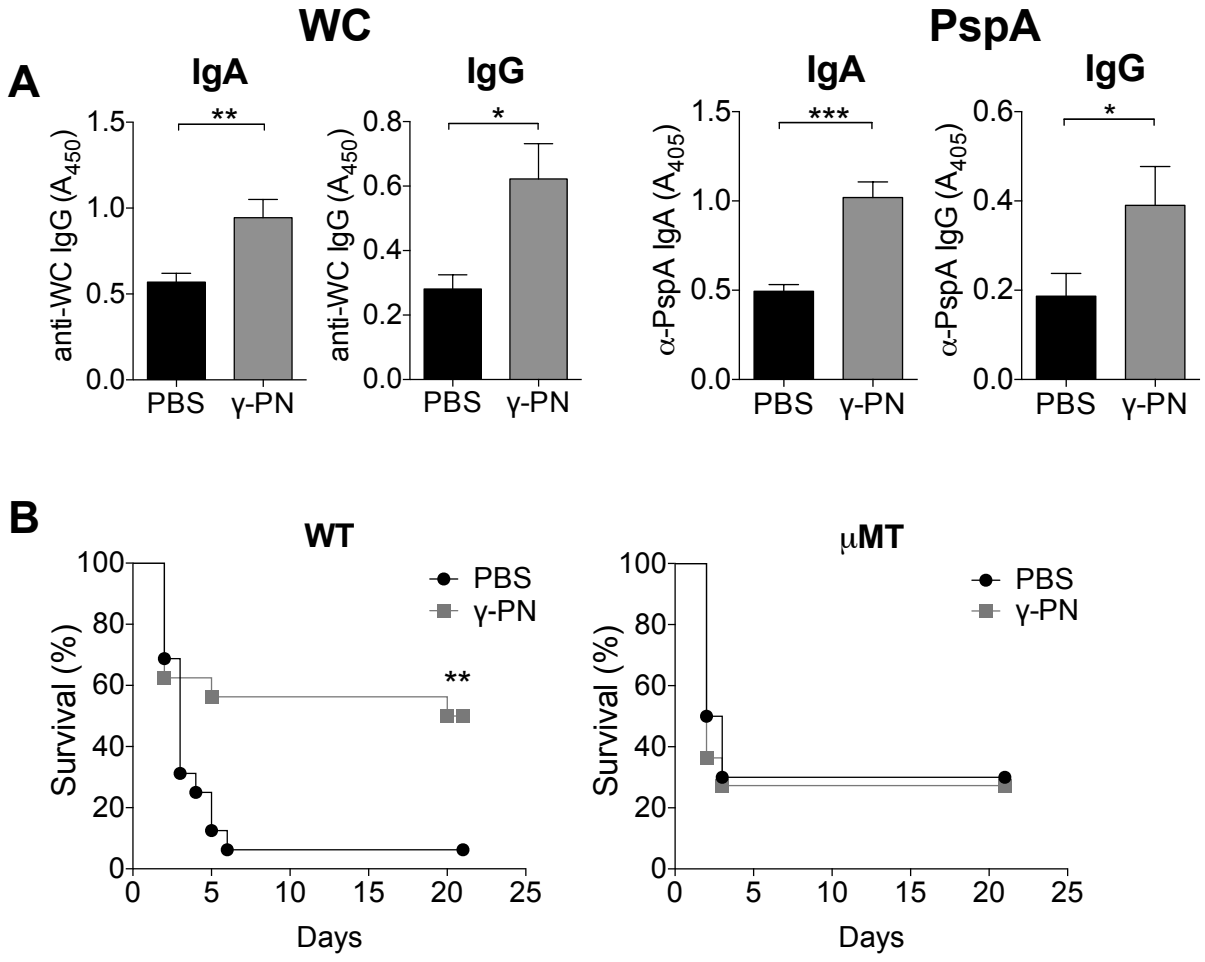


Figure 3.8: The efficacy of the γ-PN vaccine is dependent on B cell responses. (A) Sera were collected 2 weeks after the second immunization dose with γ-PN (Section 2.12) and assayed for IgA and IgG antibodies against the intact whole-cell (WC) or PspA antigen by ELISA. Data represented as mean ± SEM (n = 11). Data were analyzed using a Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B) WT or μMT mice (n = 10 ≥ per group, data pooled from 3 independent experiments) were immunized IN with 2 doses of γ-PN or PBS (Section 2.12), and 2 weeks after the second immunisation, mice were challenged IN with D39 (Section 2.14). Mice were monitored for survival for 21 days post challenge. Differences in survival rate between immunized and control mice were analyzed by a Fisher Exact test (**, $P < 0.01$).

3.2.4 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* (84, 85, 259, 360, 361). Therefore, to determine whether these cytokines could contribute to the protective efficacy of the γ-PN vaccine, mice immunized intranasally with γ-PN were treated with 3 doses of neutralising antibodies to IL-17 or IFN-γ, or isotype control antibodies during challenge with D39 as described in Section 2.15 and monitored for survival. Significant γ-PN-mediated protection against D39 challenge was seen for mice given the respective isotype control antibodies for both IFN-γ (Figure 3.9A) and IL-17 (Figure 3.9B) ($P = 0.0354$ for anti-IFN-γ isotype control group, $P = 0.0018$ for anti-IL-17 isotype control group). Importantly, γ-PN induced significant protection in immunized mice despite IFN-γ neutralisation ($P = 0.0083$) when compared to their relative control (Figure 3.9A). However, the reduced survival rate of non-immunized mice in the IFN-γ treated group compared to the isotype control group was expected due to its known role in primary host defence against pneumococcal challenge (360, 361). In contrast to IFN-γ neutralisation, γ-PN-induced protection was abolished by IL-17 neutralisation (Figure 3.9B). Moreover, IL-17 neutralisation did not influence the lethality rate of pneumococcal infection in non-immunized mice, which illustrates the role of IL-17 in the secondary immune response. These data demonstrate that the mechanisms of protection elicited by γ-PN immunisation are IL-17-dependent and in part IFN-γ-dependent.

3.2.5 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 (84). 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 colonisation mediated by mucosal immunization with a whole-cell vaccine (85). Considering the IL-17-dependent protective responses induced by γ-PN (Figure 3.9B), the mechanism underlying γ-PN-induced IL-17-dependent immunity was next investigated. Firstly, the γ-PN vaccine was analyzed for its ability to induce 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-dependent protective pneumococcal antigen SP2108, referred to as 'MalX' (86) for 72 hours. Following stimulation, cells were subjected

to intracellular cytokine staining for analysis of T helper cell subsets (Th17, Th1, Th2, Treg). The data show that stimulation of splenocytes from γ-PN immunized mice did not induce a significant increase in the frequency of Th17, Th1, Th2, or Treg cells relative to the PBS control following stimulation with either the γ-PN vaccine or MalX antigen (Figure 3.10B). These data suggest that γ-PN does not induce detectable circulating Th17 memory cell responses.

To further investigate the memory responses induced by the γ-PN vaccine, TRM cells were next analysed to determine whether these cells were related to the source of IL-17. As mentioned earlier, 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 (362, 363). Analysis of lung tissue from γ-PN immunized mice showed that these mice appear to not induce $CD4^+$ TRM cells (TRM Total, gated $CD44^{hi}CD103^+$ and were also $CD11a^{hi,}$ Figure 3.11A) relative to the PBS control, nor were there differences in the ability of these cells to make IL-17 (TRM IL-17^+) (Figure 3.11B). Collectively, these data indicate that vaccination with γ-PN does not elicit $CD4^+$ TRM cell responses.

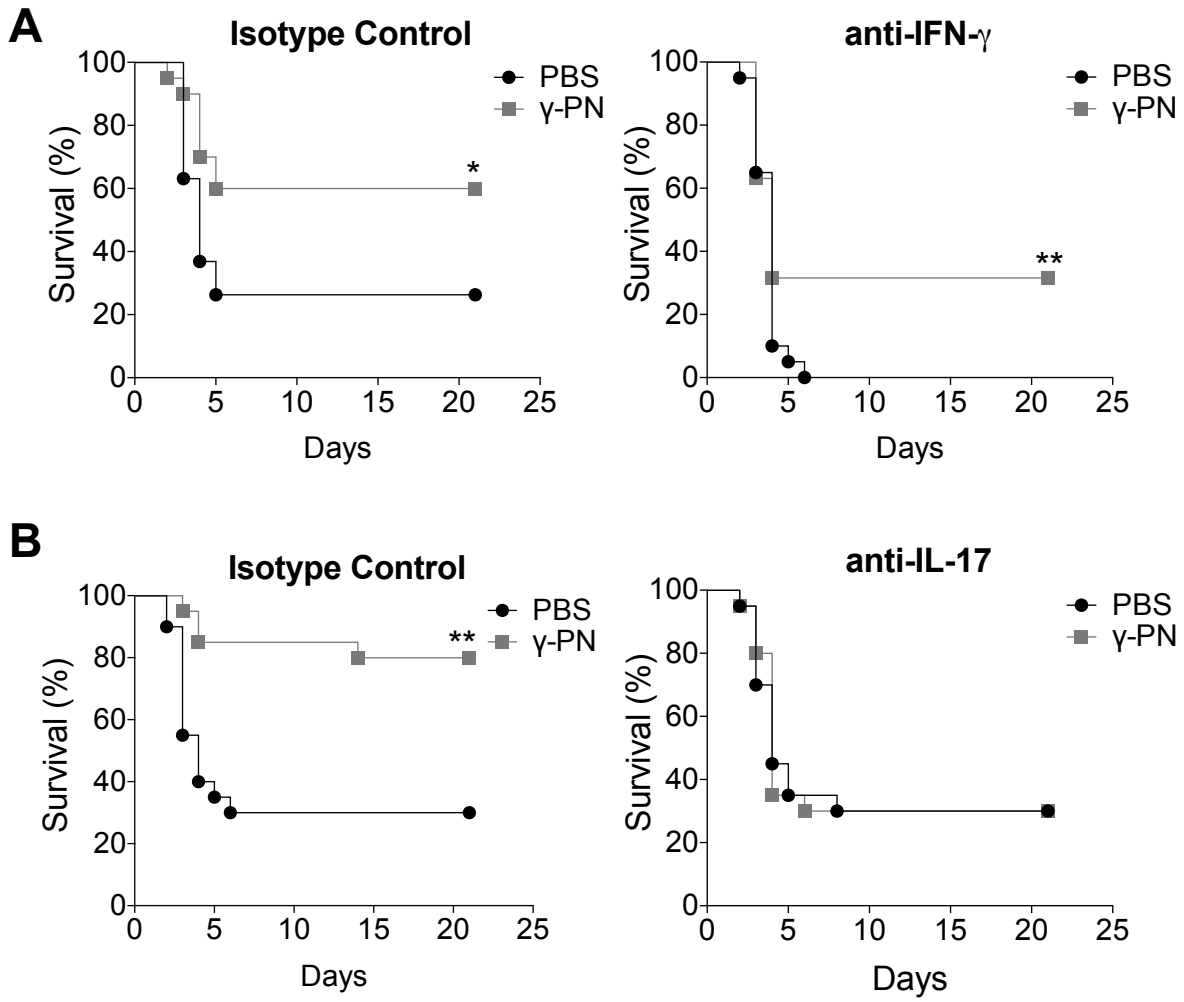


Figure 3.9: Depletion of IL-17, but not IFN-γ abrogates protection induced by γ-PN. Mice (n = 20 per group; data pooled from 2 independent experiments of n = 10) were immunized IN with 2 doses of γ-PN or given PBS (Section 2.12) and 2 weeks after the second immunisation, mice were injected IP with 200 μg of anti-IFN-γ (A), anti-IL-17 (B), or the respective isotype control, on day -1 (24 hours before challenge), day 0 (6 hours post challenge with D39) and day 2 (48 hours post challenge). Mice were monitored for survival for 21 days following challenge IN with D39 (Section 2.14). Differences in survival rates between groups of mice treated with cytokine-neutralising and control antibodies were analyzed by Fisher Exact test (*, $P < 0.05$; **, $P < 0.01$).

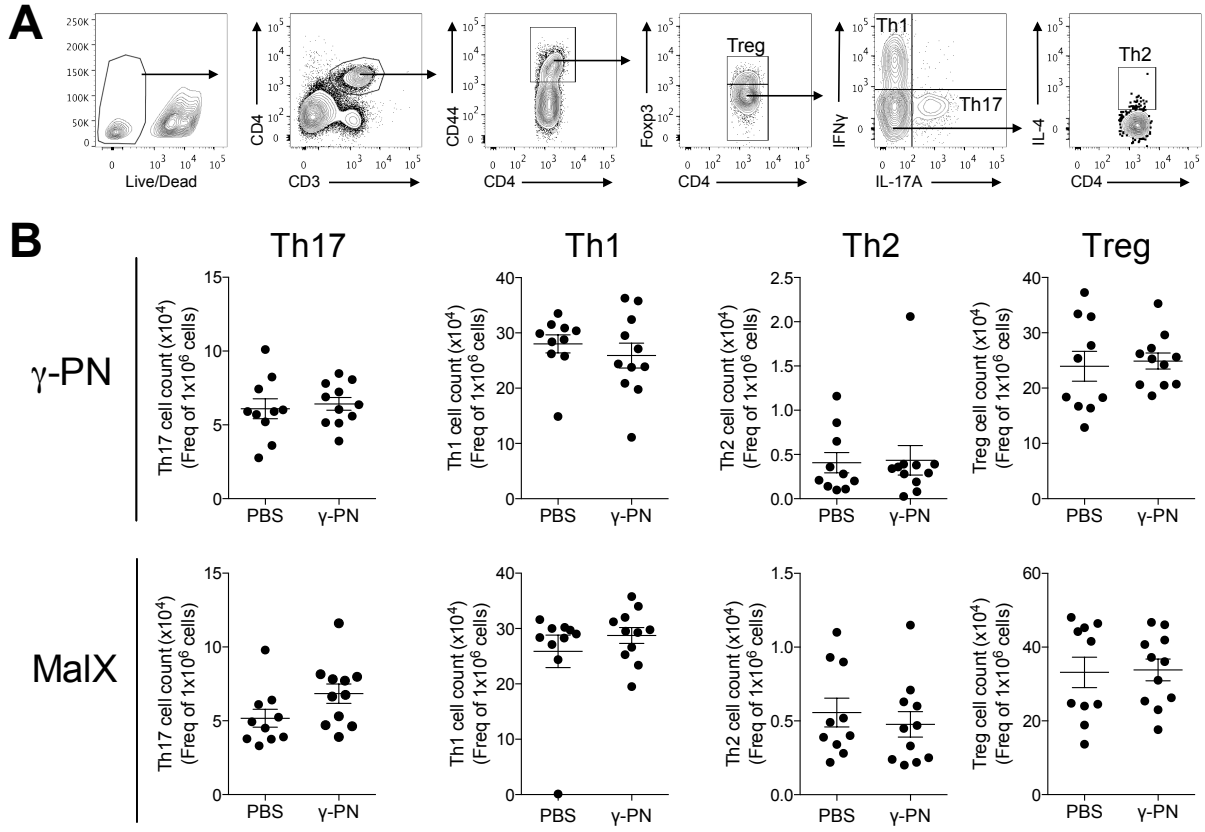


Figure 3.10: γ-PN does not induce antigen-specific Th17 cells. Mice were immunized IN with 2 doses of the γ-PN vaccine or given PBS (Section 2.12) and 2 weeks after the second immunisation, the spleens were harvested and restimulated in culture with γ-PN, or media alone (negative control) for 72 hours. (A) Representative flow cytometric data scheme for analysis of T helper cell subsets: Th1 ($CD3^+CD4^+CD44^+IFN\text{-}\gamma^+$), Th17 ($CD3^+CD4^+CD44^+IL\text{-}17^+$), Th2 ($CD3^+CD4^+CD44^+IL\text{-}4^+$) and Treg ($CD3^+CD4^+CD44^+Foxp3^+$). (B) Frequency of T cells following stimulation with the γ-PN vaccine or MalX antigen using flow cytometry. Results are presented as mean ± SEM (n =10-11 per group; data pooled from 2 independent experiments of n = 5-6 per group).

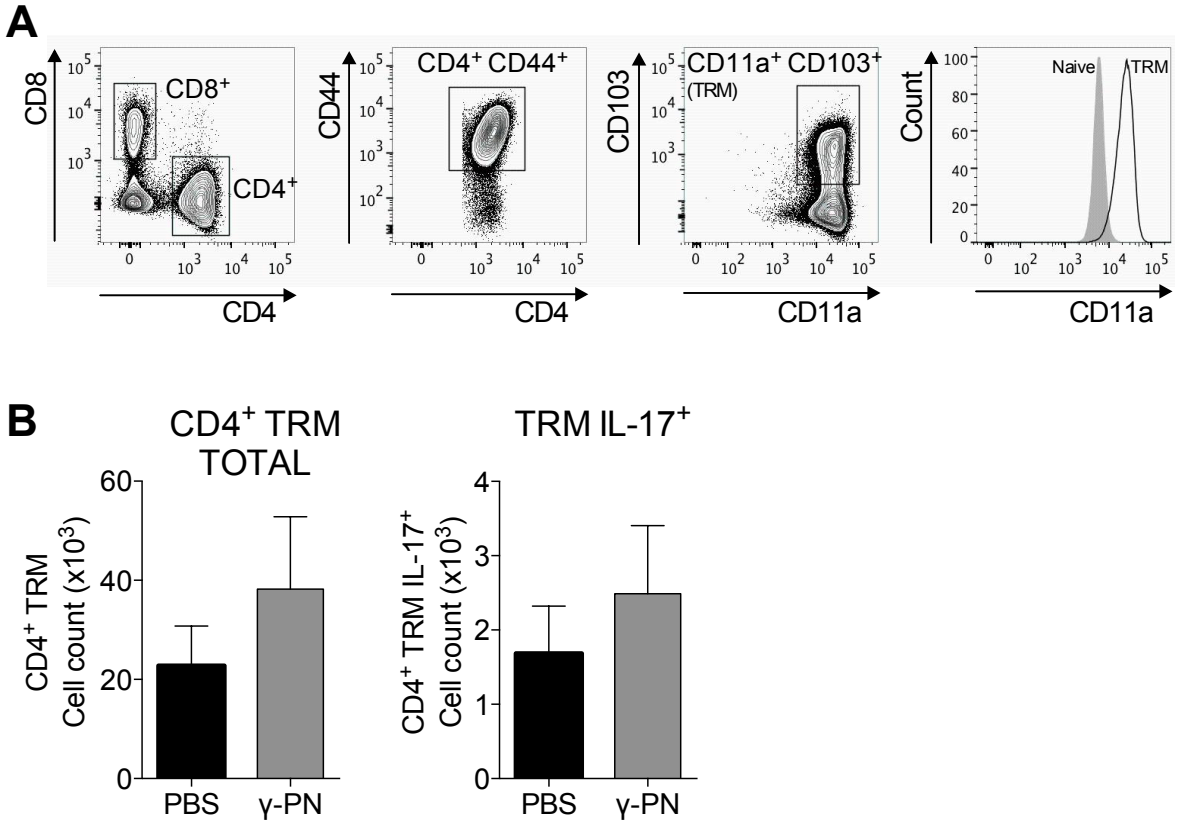
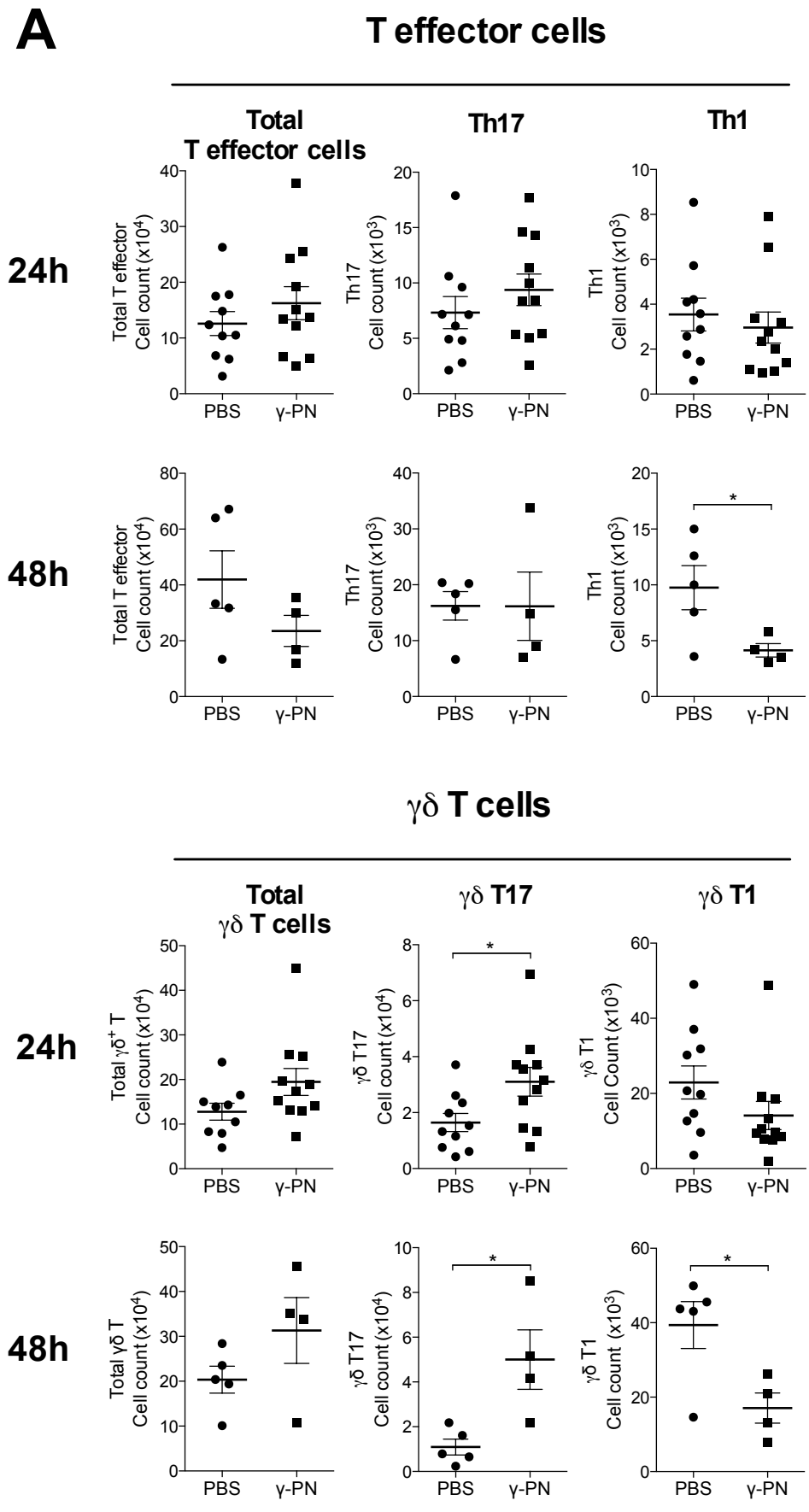


Figure 3.11: γ-PN does not induce tissue-resident memory cells. Mice were immunized IN with 2 doses of the γ-PN vaccine or given PBS (Section 2.12) and 2 weeks after the second immunisation, mice were challenged with D39 (Section 2.14). 24 hours post challenge, the lungs were harvested and analyzed for the total number of $CD4^+$ TRM cells and $CD4^+$ TRM IL-17^+ cells by flow cytometry (A) Representative flow cytometric data scheme for analysis of $CD4^+$ TRM cells ($CD4^+CD8^-CD44^+CD103^+CD11a^+$). Histogram confirming the high presence of $CD11a^+$ on TRM cells versus naïve cells. (B) Total number of $CD4^+$ TRM cells and $CD4^+$ TRM IL-17^+ cells. Results are presented as mean ± SEM (n = 10; data pooled from 2 independent experiments of n = 5).

Considering that there were no significant increases in Th17 or TRM IL-17$^+$ cells, γ-PN was next investigated for its ability to induce IL-17-producing innate immune cells. In addition to Th17 cells, non-conventional T cells such as γδ (240) and NKT cells (364) located in mucosa have been shown to elicit protective pulmonary IL-17 responses. In particular, γδT cell-derived IL-17 (γδT17) has been shown to strongly promote the recruitment of neutrophils during pneumococcal infection (69, 240). Therefore, it was hypothesized that the IL-17-dependent immunity elicited by γ-PN may be due to heightened γδT17 responses. Thus, γδ T cells and T effector cell numbers in the lungs of γ-PN immunized mice 24 and 48 hours post D39 challenge were examined. 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 hours post D39 challenge (Figure 3.12A). Similar results were observed at 48 hours for Total T effector cells and Th17. However, there was a significant decrease in Th1 cell numbers in γ-PN-immunized mice (P = 0.0452) relative to the PBS-treated controls at 48 hours post challenge. In contrast to T effector cells, vaccination with γ-PN induced significant changes in γδ T cell populations in the lung post D39 challenge (Figure 3.12A). Whilst the total number of γδ T cells in the lung of vaccinated mice was not significantly different relative to non-immunized mice, the data indicate that intranasal vaccination with γ-PN specifically enhanced γδT17 cell numbers in the lungs at 24 hours (P = 0.0297) and this was further enhanced at 48 hours post challenge (P = 0.0157). Interestingly, there was a decrease in γδT1 cell numbers in vaccinated mice at 24 hours, leading to a significant difference (P = 0.0267) detected at 48 hours relative to the control mice. Overall, these data demonstrate that γ-PN appears to promote a significant increase in γδT17 cell responses associated with a significant decrease in Th1 and γδT1 cells and no difference in Th17 cells. Thus, the data suggest that γδT17 cells may be a potential source for IL-17 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, the numbers of neutrophils and macrophages in the lungs of γ-PN-immunized mice at 24 and 48 hours post D39 challenge were investigated (Figure 3.12B). Importantly, the numbers of either cell types remain similar at 24 and 48 hours post challenge in γ-PN-immunized mice. In contrast, there was a significant increase of macrophages in the lungs of PBS control mice between 24 and 48 hours post challenge (P = 0.0122). This trend was apparent for neutrophils although it did not reach statistical significance. Nevertheless the number of phagocyte cells at 48 hours was increased in PBS control mice in comparison to γ-PN immunized mice.


A
T effector cells
Total T effector cells
Th17
Th1
24h
48h
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Th17 Cell count (x10³)
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PBS
γ-PN
*
γδ T cells
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γδ T17
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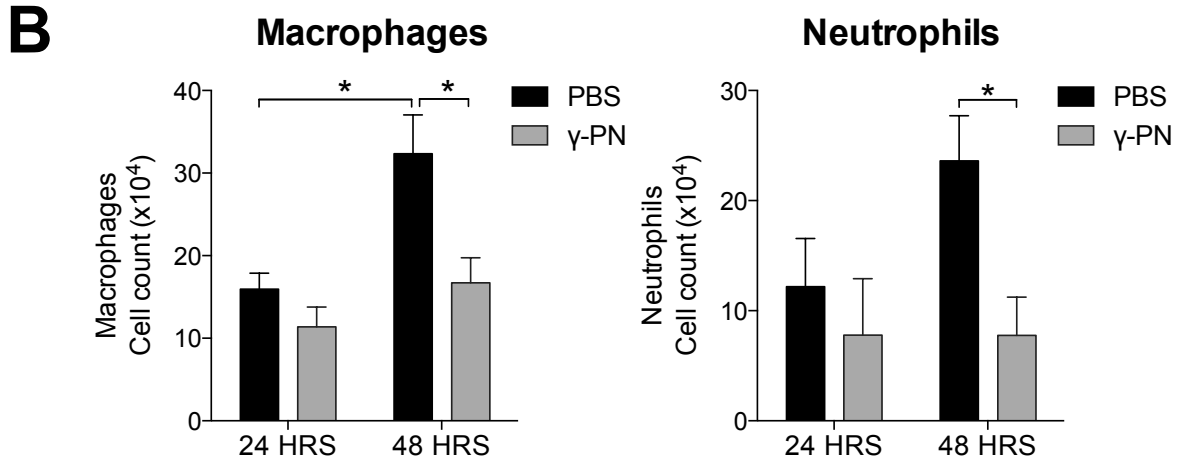


Figure 3.12: Vaccination with γ-PN induces IL-17 via γδ T cells, not CD4$^+$ T cells. Mice were immunized IN with 2 doses of γ-PN or given PBS (Section 2.12), and 2 weeks after the second immunisation, mice were challenged IN with D39 (Section 2.14). Lungs were harvested at 24 and 48 hours post challenge and analyzed for the total number of the following cells by flow cytometry. (A) T effector cells: Total T effector cells ($CD3^+CD4^+CD44^+$), Th17 ($CD3^+CD4^+CD44^+IL\text{-}17^+$), Th1 ($CD3^+CD4^+CD44^+IFN\text{-}\gamma^+$); (B) γδ T cells: Total γδ T cells ($CD3^+CD44^+\gamma\delta TCR^+$), γδT1 ($CD3^+CD44^+\gamma\delta TCR^+IFN\text{-}\gamma^+$), γδT17 ($CD3^+CD44^+\gamma\delta TCR^+IL\text{-}17^+$); and (C) phagocytic cells: macrophages ($CD45^+CD11b^+Gr1^{lo}F4/80^+$) and neutrophils ($CD45^+CD11b^+Gr1^{hi}$). Results are presented as mean ± SEM; *, $P < 0.05$ (Student's *t*-test). Results are pooled from 2 independent experiments ($n \geq 9$) for the 24 hour time point. For the 48 hour time point, $n \geq 4$.

3.3 Discussion

In recent years there has been a considerable focus on developing pneumococcal vaccines that are serotype-independent, cost effective and capable of providing protective immunity in people of all ages. The success of using gamma-irradiation to inactivate bacteria has been demonstrated for *Rickettsia* (334), *Brucella abortus* (336) and *Listeria monocytogenes* (335). In this study gamma-irradiated, un-encapsulated pneumococcal whole-cells were administered to maximise 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 pneumococcus in humans. During the initial stages of pneumococcal colonization in the nasopharynx, the capsular polysaccharide is down-regulated and there is increased exposure of cell wall teichoic acids and surface proteins, which facilitate attachment to the mucosa (365, 366). Thus, intranasal delivery of an un-encapsulated whole-cell vaccine is expected to elicit enhanced immunity towards these sub-capsular structures. Gamma-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 *Listeria monocytogenes* (335) and chemical or UV inactivation methods as shown for Influenza (329).

This study demonstrates that intranasal immunisation with the un-adjuvanted γ-PN vaccine provides significant serotype-independent protection against sepsis caused by D39 and P9, and focal pneumonia caused by EF3030. The vaccine strain irradiated at 12 kGy on DI (γ-PN) was chosen as the optimal vaccine preparation as it was completely sterile with no detectable damage to the cellular structure or membrane integrity, and was observed to have high immunogenicity. Interestingly, higher doses of gamma-irradiation appeared to affect the membrane integrity and immunogenicity of the vaccine preparations despite the limited effect on the morphology of the bacterial cells. The process of gamma-irradiation mainly inactivates pathogens through direct actions on nucleic acids. However, it can also have indirect actions with solutes i.e. with water molecules and oxygen which result in the generation of free radicals (358). Therefore, higher doses of gamma-rays may increase free radical generation that contributes to inactivation of the pathogen but also causing limited damage, particularly to proteins. This could have affected the antigenic structures and may explain why higher doses above 12 kGy were not as immunogenic. In addition, it could also explain why the

bacterial envelope was not as intact as observed with Gram staining in comparison to the 12 kGy preparation.

To investigate the underlying mechanism for serotype-independent protection induced by γ-PN, the role of B and T cells in acquired immunity was evaluated. Lack of protective immunity in vaccinated μMT mice illustrated the significant contribution of B cell responses. Generally, antibodies against capsular and sub-capsular antigens are important for complement fixation and opsonisation in systemic clearance of pneumococci (1). Thus, the 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 pneumococcal-specific antibody responses to the WC strain and PspA protein. In particular, humoral responses against PspA help to prevent interference with complement-mediated opsonisation, 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 (367). Indeed, Th17 cell responses have been shown to be important for abrogation of the initial stages of bacterial colonization (82, 85, 368), 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 (85). In the present study intranasal vaccination with γ-PN did not induce antigen-specific Th17 responses, which is a probable explanation for why there was no protection observed in the EF3030 colonization model. Nevertheless, neutralisation 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 immunisation resulted in enhanced levels of pulmonary γδT17 cells. These cells represent one of the major innate sources of IL-17 during infection, which plays critical roles in protection against extracellular pathogens such as *S. pneumoniae*. In addition, γδT cell-deficient mice have low survival rates that were shown to be associated with higher bacterial loads and reduced neutrophilia following pneumococcal infection (69, 240). 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* (84). Therefore, it is feasible that γ-PN immunisation may promote neutrophilia through enhanced γδT17 responses, which combines with

opsonising antibodies to clear pneumococcal infection. Furthermore, although the data shown indicates a clear and prominent role for IL-17 in vaccine-elicited protection, it also appears that IFN-γ may be playing a 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* (360). The data indicate that IFN-γ plays a more prominent protective role compared to IL-17 during primary infection.

Nevertheless, whilst the data illustrate the significantly increased number of γδT17 cells in the lung of vaccinated animals, other innate IL-17 producers such as NKT cells may also be involved. An emerging concept in γδT cell biology is the ability of these cells to adopt 'memory'-like properties. Oral *Listeria monocytogenes* (Lm) infection induces a subset of protective γδT cells that preferentially expand upon oral, but not intravenous Lm re-infection or oral *Salmonella* infection (369). Similar observations have also been made in a model of *S. aureus* infection (249). Furthermore, in imiquimod-induced psoriasis inflammation, γδT17 cells have been shown to redistribute into non-inflamed skin tissue, are activated more rapidly via IL-1β and accelerate secondary skin inflammation upon re-exposure to imiquimod (370). In the present study, it is not clear whether γ-PN directly induces 'memory'-like γδT17 cell responses or whether there is a γ-PN-induced adaptive response that leads to enhanced γδT17 recruitment or expansion/sensitivity within the lung. More detailed studies will be required to elucidate the mechanisms of γ-PN-induced γδT17 cell responses.

Furthermore, immunological analysis of phagocytic cells at 24 and 48 hours post D39 challenge showed no difference in total levels of macrophages and neutrophils in γ-PN immunized mice. In addition, levels of phagocytic cells at 48 hours were reduced relative to PBS control mice. The stable level of inflammation observed in vaccinated mice could imply that the majority of pneumococci are cleared within 48 hours, compared to 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 (371). Therefore, rapid bacterial clearance and reduced influx of phagocytic cells may underpin the efficacy of γ-PN vaccine.

In addition, there was a greater increase in the number of γδT17 cells in γ-PN-immunized mice at 48 versus 24 hours post challenge. This suggests that γδ-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 beta defensin 2 (372), which has been reported to work synergistically with lysozymes to inhibit the growth of *S. pneumoniae* (373). 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 γδT cells, thereby exacerbating infection (239). Therefore, γ-PN-induced γδT17 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.

The data also illustrate limited efficacy for γ-PN against nasopharyngeal carriage compared to previous reports using an ethanol-killed whole-cell pneumococcal vaccine, that demonstrated protective efficacy against pneumococcal carriage via antibody-independent, Th17-driven immunity (85). However, experience with pneumococcal conjugate vaccines has shown that elimination of carriage may be a "double edged sword", whereby the benefits of blockading 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* (374). An important additional consideration is that efficacy of previous experimental pneumococcal vaccines targeted for mucosal administration, such as protein combination (119, 355) and whole-cell (133) vaccines, has been dependent on formulation with strong mucosal adjuvants, such as CT, which are unlikely to be approved for human use. In contrast, the γ-PN vaccine is non-adjuvanted and still elicits significant protection. It is proposed that gamma-irradiation of pneumococcal whole-cells is associated with reduced damage to antigenic epitopes which may facilitate the ability of the gamma-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.

Intranasal immunization is highly advantageous due to the fact that it is relatively safe, painless, needle free and a more acceptable vaccination approach for the public. It is also easily deliverable and decreases the risk of disease transmission that can occur through the use of syringes (219).

In summary, this study describes the development of a non-adjuvanted gamma-irradiated whole-cell pneumococcal vaccine that elicits serotype-independent protection against both pneumococcal sepsis and focal pneumonia. The efficacy of the γ-PN vaccine was shown to be dependent on both B cell and IL-17 responses. In particular, to my knowledge this is the first report known to demonstrate the induction of vaccine-induced innate-derived IL-17-responses. The induction of γδT17 cells by γ-PN vaccination may provide a strategic immunisation approach to protect, not only against single pneumococcal challenge but also against super-infection by other pathogens.

CHAPTER 4

The role of adjuvants in γ-PN vaccine efficacy

4.1 Introduction

In recent years, intranasal delivery of antigens has become a more acceptable vaccination approach relative to the parenteral route (375). Aside from the advantage that intranasal vaccination is non-invasive, it allows superior induction of mucosal immunity, which is particularly important for respiratory pathogens such as *S. pneumoniae* (376). However, given the poor immune responses often observed with intranasally administered vaccines and the concerns with live-attenuated strains in reverting back to their virulent form, there has been a focus on combining inactivated vaccines with mucosal adjuvants to enhance their immunogenicity (265). In particular, enterotoxins such as CT and LT have been studied intensively as mucosal adjuvants, and have been reported to induce potent systemic and mucosal antibody responses against co-administered antigens (276). The success of CT as an adjuvant has been attributed to many roles (Section, 1.6.1), including the ability of the toxin to enhance transepithelial influx of the vaccine into nasal mucosa, thereby promoting APC activation and subsequent presentation of antigens (279, 377). Previously, studies focusing on the development of pneumococcal intranasal vaccines have employed CT as an adjuvant to enhance immunogenicity and efficacy of tested vaccines (117, 133, 286). Prior work related to an ethanol-killed whole-cell pneumococcal vaccine revealed the profound efficacy against colonisation when administrated with CT as an adjuvant. In addition, administration of pneumococcal whole-cells with CT has also been shown to induce IL-17-dependent immunity (85, 133, 135). Despite the reported efficacy, there is a significant safety concern regarding use of enterotoxins to be employed as mucosal adjuvants due to their associated toxicity, and therefore they have not be approved for human use (287, 378, 379).

In recent years, delivery systems such as chitosan and its derivatives have been employed as possible mucosal adjuvants. Chitosan is derived from the N-deacetylation of chitin, a natural polysaccharide which constitutes the exoskeleton of arthropods. It is nontoxic and non-allergenic, and therefore has been considered a promising candidate for the delivery of mucosal vaccines (380-383). The mechanisms underlying the effectiveness of chitosan as an adjuvant are still not fully understood, but the presence of highly protonated amino groups confers a positive charge on the polysaccharide, which allows effective adherance to muscosal surfaces (384). Furthermore, it has the ability to open tight junctions between cells and therefore it has also been proposed to enhance uptake of the delivered antigen into the nasal associated lymphoid tissue (376, 385, 386). Previous studies have shown that intransal

delivery of chitosan enhances mucosal immune responses, specifically humoral responses (387, 388) and Th2 immunity (389, 390). However, administration of chitosan via subcutaneous routes has been associated with induction of Th1 immunity and Th17 responses when combined with the TL4 agonist CpG (391, 392). Importantly, intranasal immunisation with chitosan-DNA nanoparticles expressing pneumococcal surface protein PsaA has been reported to induce protective mucosal and systemic immune responses, which were able to provide significant protection against pneumococcal colonization (393). However, the adjuvant activity of chitosan has not been tested previously with pneumococcal whole-cell vaccines.

It was earlier shown in Chapter 3 that intranasal vaccination with γ-PN without an adjuvant conferred significant serotype-independent protection, which appeared to be reliant on B cells and the induction of innate-derived IL-17. Therefore, to determine whether the immunogenicity and protective efficacy of the γ-PN vaccine can be enhanced, intranasal adjuvants CT and mannosylated chitosan (MC) were investigated.

4.2 Results

4.2.1 Intranasal vaccination with γ-PN + CT elicits optimal protection against pneumococcal challenge.

As shown in Chapter 3, intranasal administration of the γ-PN vaccine without an adjuvant was capable of eliciting serotype-independent protection against lethal pneumococcal challenge. In general, the protective efficacies of pneumococcal whole-cell vaccines have been previously investigated using CT as a mucosal adjuvant (133, 135, 286). Therefore, the impact of CT on the efficacy of the γ-PN vaccine was investigated. Mice were vaccinated intranasally with 2 doses of γ-PN or γ-PN and CT (γ-PN + CT). Control animals were treated with CT or PBS. 2 weeks after the second vaccination dose, mice were challenged with *S. pneumoniae* serotype P9, which is a different strain lineage relative to the γ-PN vaccine strain. As shown in Figure 4.1, following P9 challenge, mice vaccinated with γ-PN + CT elicited significant protection relative to the CT control as illustrated by an increase in survival time (P = 0.0066) and survival rate (P =0.0034) (Figure 4.1A & B). Importantly, survival rates of γ-PN + CT vaccinated mice were higher (100%) in comparison to mice vaccinated with γ-PN alone (70%), although this difference did not reach statistical significance. Mice vaccinated with un-adjuvanted γ-PN also showed protection against P9

challenge as illustrated by longer survival times and higher survival rates relative to the PBS control, but this did not reach statistical significance.

Studies employing CT as an adjuvant for pneumococcal vaccines have also reported the protective efficacy of these vaccines against nasopharyngeal carriage (82, 117, 133, 286). Therefore, the efficacy of vaccination with γ-PN + CT against local colonization in the nasopharynx was also investigated. Post immunization, mice were colonized with EF3030, as described for the colonization model (Section 2.14). Interestingly, there appeared to be no reduction in bacterial counts in the nasopharynx of mice vaccinated with γ-PN + CT or γ-PN alone relative to the respective controls (Figure 4.2A &B). Overall, these data show that intranasal vaccination with γ-PN + CT elicits optimal serotype-independent protection relative to immunisation with un-adjuvanted γ-PN. However, the use of CT as an adjuvant does not appear to have an impact on nasopharyngeal colonisation.

4.2.2 Intranasal vaccination with γ-PN + CT enhances pneumococcal-specific antibody levels and induces Th17 cells.

Considering the higher survival percentage observed following vaccination with γ-PN + CT in comparison to vaccination with γ-PN alone, the underlying immunological mechanisms associated with the observed enhanced protection were next investigated. Given the known role of CT in enhancing humoral responses to co-administered antigens, the impact of CT administration on pneumococcal-specific antibody levels was evaluated. 2 weeks post the second vaccination dose, serum samples were collected from vaccinated and control mice and analyzed for pneumococcal specific IgG and IgA levels by ELISA. As shown in Figure 4.3, there were significantly enhanced IgG and IgA titres in the sera from γ-PN + CT vaccinated mice relative to sera from γ-PN vaccinated mice. This suggests that enhanced antibody responses may contribute to higher survival rates observed following vaccination with γ-PN + CT.

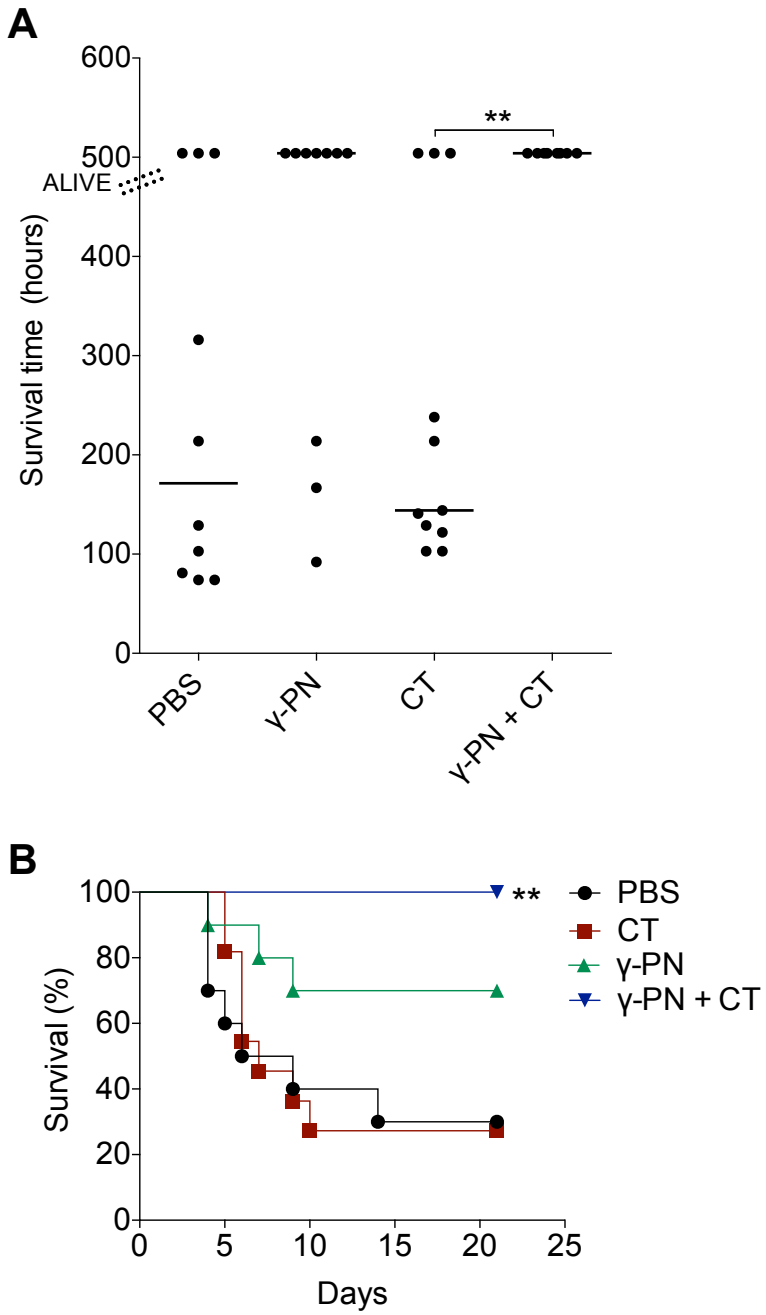


Figure 4.1: Intranasal vaccination with γ-PN + CT enhances the protective efficacy of the γ-PN vaccine. Mice (n = 10) were immunized IN with 2 doses of γ-PN or γ-PN + CT and control groups were treated with CT or PBS respectively (Section 2.12-13). 2 weeks after the second immunisation, mice were challenged IN under anaesthesia with P9 (Section 2.14). Mice were monitored for a period of 21 days and survival time (A) and survival rates (B) are recorded. Data were analyzed using a Mann-Whitney *U*-test (**, $P < 0.01$) (survival time) and a Fisher exact test (**, $P < 0.01$) (survival rate).

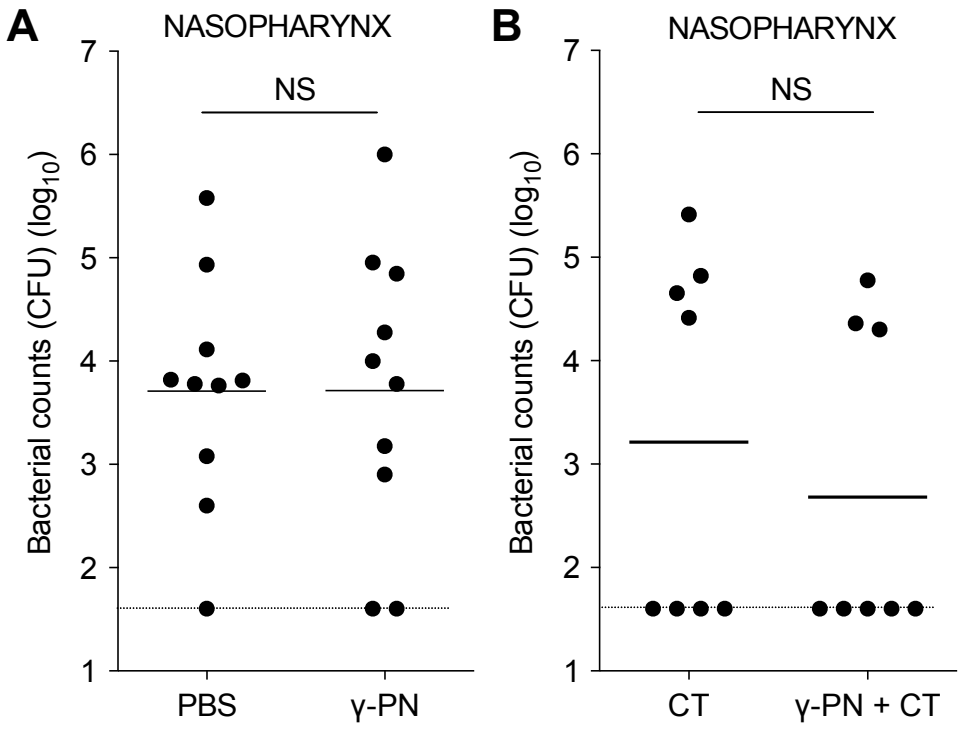


Figure 4.2: Intranasal vaccination with γ-PN + CT does not elicit protection against nasopharyngeal colonization. Mice (n = 10) were immunized IN with 2 doses of γ-PN or γ-PN + CT and control groups were treated with PBS or CT respectively (Section 2.12-13). 2 weeks after the second immunisation, mice were challenged with EF3030 without anaesthesia (colonization model) (Section 2.14). Bacterial counts in the nasopharynx at 7 days post inoculation are shown. Dotted line represents detection limit. *Please note that data related to γ-PN vaccinated mice have been presented previously in Figure 3.7. However, mice vaccinated with either γ-PN or γ-PN + CT were performed at the same time and therefore presented here for appropriate comparison.*

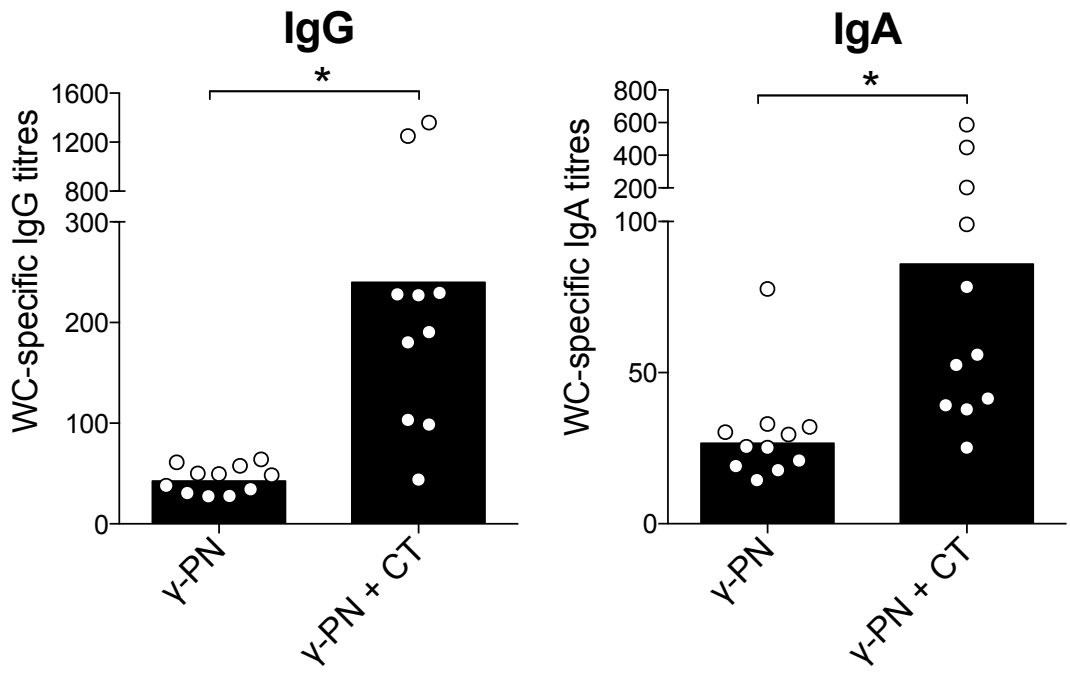


Figure 4.3: Intranasal vaccination with γ-PN + CT is associated with enhanced pneumococcal-specific antibody titres. Mice (n = 11) were immunized IN with 2 doses of γ-PN or γ-PN + CT and control groups were treated with PBS or CT respectively (Section 2.12-13). 2 weeks after the second immunisation, sera were collected and tested for IgG and IgA-specific antibodies against the whole-cell vaccine strain by ELISA. Geometric mean for antibody titre is shown. Data were analyzed using a Student's unpaired *t*-test (*, $P < 0.05$).

Given the importance of Th17 responses in pneumococcal infection and the role of Th17 in protective immunity induced by pneumococcal vaccines employing CT as an adjuvant (84, 85), the T helper cell memory responses induced following immunisation with γ-PN + CT were investigated. Mice were vaccinated intranasally with 2 doses of γ-PN or γ-PN + CT. 2 weeks post second vaccination dose, spleens were harvested and splenocytes were stimulated for 72 hours with either γ-PN or MalX. Following stimulation, culture supernatants were analyzed for levels of IL-17 and IFN-γ by ELISA and the frequency of T helper cell subsets were analyzed by flow cytometry. *It is important to note that vaccination with γ-PN + CT or γ-PN alone was carried out at the same time and that data related to vaccination with γ-PN alone was previously presented in Figure 3.10. Thus, please refer to Figure 3.10 regarding immunisation with γ-PN alone*. As shown in Figure 4.4, significant increases in the frequency of pneumococcal-specific Th17 cells were detected in splenocytes from γ-PN + CT vaccinated mice relative to the CT treated control following *ex vivo* stimulation with either the γ-PN or MalX antigen (Figure 4.4B). Interestingly, there also appeared to be a significant decrease in the frequency of Th1 cells upon stimulation with the MalX antigen. Analysis of culture supernatants also showed significantly higher levels of IL-17 and IFN-γ after stimulation with γ-PN or MalX antigen in splenocyte cultures from γ-PN + CT vaccinated mice relative to the CT treated control (Figure 4.4C). Overall, these data illustrate that intranasal vaccination with γ-PN + CT promotes the induction of pneumococcal-specific Th17 memory cells.

To look further into the type of memory responses induced when CT is employed as an adjuvant with the γ-PN vaccine, the lungs were evaluated for potential development of TRM cells. Lungs of immunized mice were harvested and examined for the levels of $CD4^+$ TRM and $CD8^+$ TRM. As shown in Figure 4.5, there was a significant increase in the number of $CD4^+$ and $CD8^+$ TRM cells in mice vaccinated with γ-PN + CT relative to vaccination with γ-PN alone. However, there did appear to be an increase in the number of $CD4^+$ TRM cells following vaccination with γ-PN + CT when compared to the CT control group, although this did not reach statistical significance. Importantly, mice vaccinated with γ-PN alone did not show any increase in $CD4^+$ or $CD8^+$ TRM cells relative to PBS control group. Interestingly, mice treated with CT appeared to show higher levels of $CD4^+$ and $CD8^+$ TRM cells relative to the PBS control group. However, the observed increase in TRM cells in the CT control group is expected to be non-specific to *S. pneumoniae*. Overall, these data suggest that immunisation with γ-PN + CT promotes the development of $CD4^+$ and $CD8^+$ TRM cells.

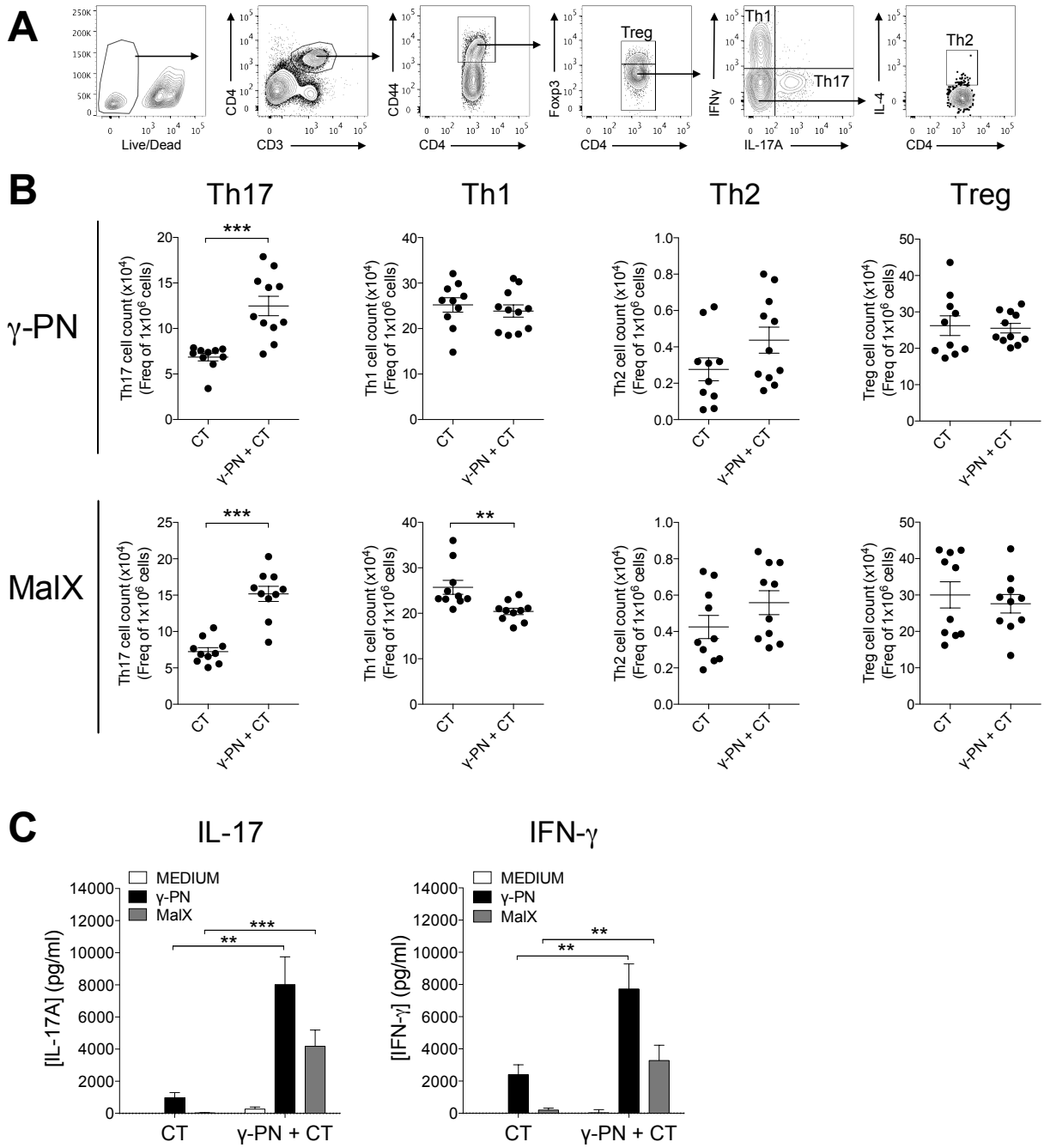

A
Live/Dead
CD3
CD4
CD4
CD44
CD4
Foxp3
Treg
CD4
IFNγ
IL-17A
Th1
Th17
IL-4
CD4
Th2
B
Th17
Th1
Th2
Treg
γ-PN
MalX
Th17 cell count (x10⁴) (Freq of 1x10⁶ cells)
Th1 cell count (x10⁴) (Freq of 1x10⁶ cells)
Th2 cell count (x10⁴) (Freq of 1x10⁶ cells)
Treg cell count (x10⁴) (Freq of 1x10⁶ cells)
CT
γ-PN + CT
***
**
C
IL-17
IFN-γ
MEDIUM
γ-PN
MalX
[IL-17A] (pg/ml)
[IFN-γ] (pg/ml)
CT
γ-PN + CT

Figure 4.4: Intranasal vaccination with γ-PN + CT induces antigen-specific Th17 cells. Mice were immunized IN with 2 doses of the γ-PN + CT or treated with CT alone as a control (Section 2.12-13). 2 weeks after the second immunisation, the spleens were harvested and restimulated in culture with γ-PN, MalX or media alone (negative control) for 72 hours. (A) Representative flow cytometric data scheme for analysis of T helper cell subsets: Th1 ($CD3^+CD4^+CD44^+IFN\text{-}\gamma^+$), Th17 ($CD3^+CD4^+CD44^+IL\text{-}17^+$), Th2 ($CD3^+CD4^+CD44^+IL\text{-}4^+$) and Treg ($CD3^+CD4^+CD44^+Foxp3^+$). (B) Frequency of T cells following stimulation with the γ-PN vaccine or MalX antigen using flow cytometry. (C) Cells stimulated in culture were centrifuged and the supernatants were collected and analyzed for the level of IL-17 and IFN-γ by ELISA. Results are presented as mean ± SEM (n = 10; data pooled from 2 independent experiments of n = 5). Data were analyzed using a Student's *t*-test, (**, $P < 0.01$; ***, $P < 0.001$).

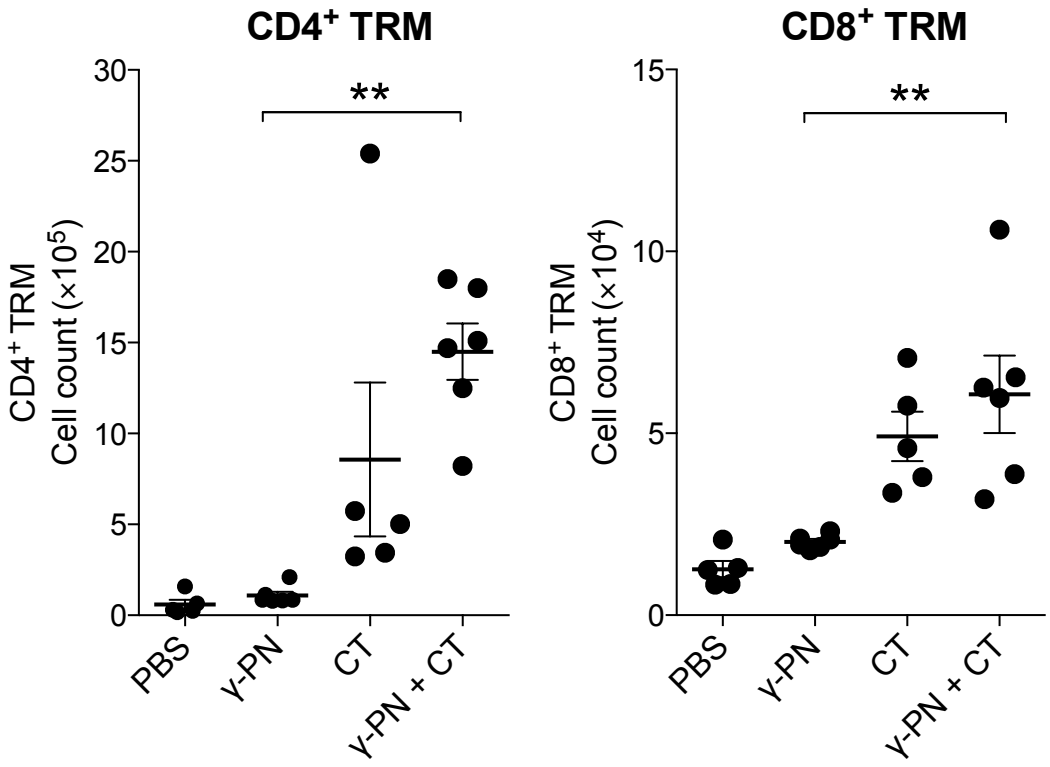


Figure 4.5: Intranasal vaccination with γ-PN + CT promotes the development of TRM cells. Mice (n ≥ 6) were immunized IN with 2 doses of γ-PN or γ-PN + CT and control groups were treated with PBS or CT respectively (Section 2.12-13). 2 weeks after the second immunisation, the lungs were harvested and analyzed for the total number of $CD4^+$ TRM ($CD4^+CD8^-CD44^+CD103^+$) and $CD8^+$ TRM cells ($CD8^+CD4^-CD44^+CD103^+$) by flow cytometry. Results are presented as mean ± SEM. Data were analyzed using a One-way ANOVA, (**, $P < 0.001$; ***, $P < 0.001$).

4.2.3 Intranasal vaccination with γ-PN + MC

Although the γ-PN vaccine and other pneumococcal vaccines incorporating CT as an adjuvant are quite effective at eliciting protective immunity against pneumococcal challenge, clinical administration of CT is not expected to be approved due to problems associated with toxicity. Therefore, researchers have been investigating alternative adjuvants for intranasal administration. Chitosan and its derivatives have been widely studied and considered promising adjuvant candidates for mucosal vaccines. Therefore, the effect of MC on γ-PN vaccine immunogenicity was next investigated. Mice were vaccinated intranasally with 2 doses of γ-PN + MC (20 μg) or γ-PN alone. Mice treated with the respective concentration of MC or PBS served as controls. 2 weeks after the second vaccination dose, mice were challenged with D39 (1×10^6 CFU/mouse) and monitored for survival. The data show that vaccination with γ-PN alone elicited significant protection against D39 challenge in comparison to the non-vaccinated PBS-treated controls ($P = 0.0349$ based on survival rates) (Figure 4.6B). There was also an increase in median survival time, although this did not reach statistical significance ($P = 0.0537$) (Figure 4.6A). Mice vaccinated with γ-PN + MC (20 μg) appeared to show longer survival times and higher survival rates relative to the MC control, but this did not reach statistical significance. Interestingly, control mice treated with MC alone showed comparable, but a slightly reduced level of protection to that observed with the vaccinated groups. Overall this data demonstrate that immunisation with γ-PN + MC did not alter the protective efficacy of the γ-PN vaccine. It also shows that 20 μg/mouse of MC induced non-specific immunity.

Considering the 60% survival rate observed in mice treated with 20 μg/mouse of MC (Figure 4.6), for the next experiment the MC concentration was reduced (from 20 μg to 6 μg) and the challenge dose of D39 was increased to evaluate whether MC had an impact on the protective efficacy of γ-PN. Mice were vaccinated intranasally with 2 doses of γ-PN or γ-PN + MC (6 μg) and mice treated with the relative concentration of MC or PBS alone served as controls. Two weeks after the second vaccination, mice were challenged with an increased challenge dose of D39 (2.5×10^6 CFU) and monitored for survival. As shown in Figure 4.7, employing a lower dose of MC considerably reduced the non-specific immunity induced by MC, as survival time and survival percentages remained similar to that of PBS treated mice. Interestingly, vaccination with γ-PN alone did not elicit significant protection relative to the PBS control. Importantly, mice vaccinated with γ-PN + MC appeared to have higher median

survival times and survival percentages relative to mice vaccinated with γ-PN alone and the corresponding control (MC). However, these results did not reach statistical significance.

To analyze the effect of MC on the immunogenicity of the γ-PN vaccine, serum samples from immunized mice were tested for pneumococcal-specific IgG or IgA titres using the whole-cell antigen. As shown in Figure 4.8, intranasal vaccination with γ-PN + MC did not significantly alter IgG or IgA-specific pneumococcal titres. Although there appeared to be elevated levels of IgA antibodies this did not reach statistical significance. Therefore, the data suggest that MC has limited adjuvant activity at low concentrations and high concentrations of MC may induce non-specific immune responses.

4.3 Discussion

Adjuvants are usually employed to enhance vaccine immunogenicity and to promote long lasting immunity. Data presented in Chapter 3 show the effectiveness of an intranasally administered un-adjuvanted gamma-irradiated pneumococcal whole-cell vaccine in providing serotype-independent protection. Interestingly, the vaccine promoted IL-17 through innate mechanisms, a distinct immune phenotype from what has been reported recently with conventional pneumococcal whole-cell vaccines. To investigate the possibility of enhancing the immunogenicity of γ-PN vaccine, CT and MC mucosal adjuvants were investigated.

Mucosal pneumococcal whole-cell vaccines adjuvanted with CT have been shown to induce protective immunity against colonization and invasive disease (133, 135, 286). The efficacy of CT adjuvanted pneumococcal whole-cell vaccines in reducing nasopharyngeal carriage has been reported to be dependent on Th17 cells for neutrophil-mediated clearance of colonizing bacteria (82, 85). The data presented in this study show that intranasal administration with γ-PN + CT resulted in optimal serotype-independent protection against challenge with P9, as a sepsis model. This was illustrated by higher survival rates observed for mice immunized with γ-PN + CT in comparison to mice vaccinated with non-adjuvanted γ-PN. Surprisingly, immunisation with γ-PN + CT did not confer protection against nasopharyngeal colonization, which is comparable with the lack of protection observed when mice are vaccinated with γ-PN alone. However, analysis of T cell memory responses from *ex vivo* stimulation experiments revealed that immunisation with γ-PN + CT induced significant levels of Th17

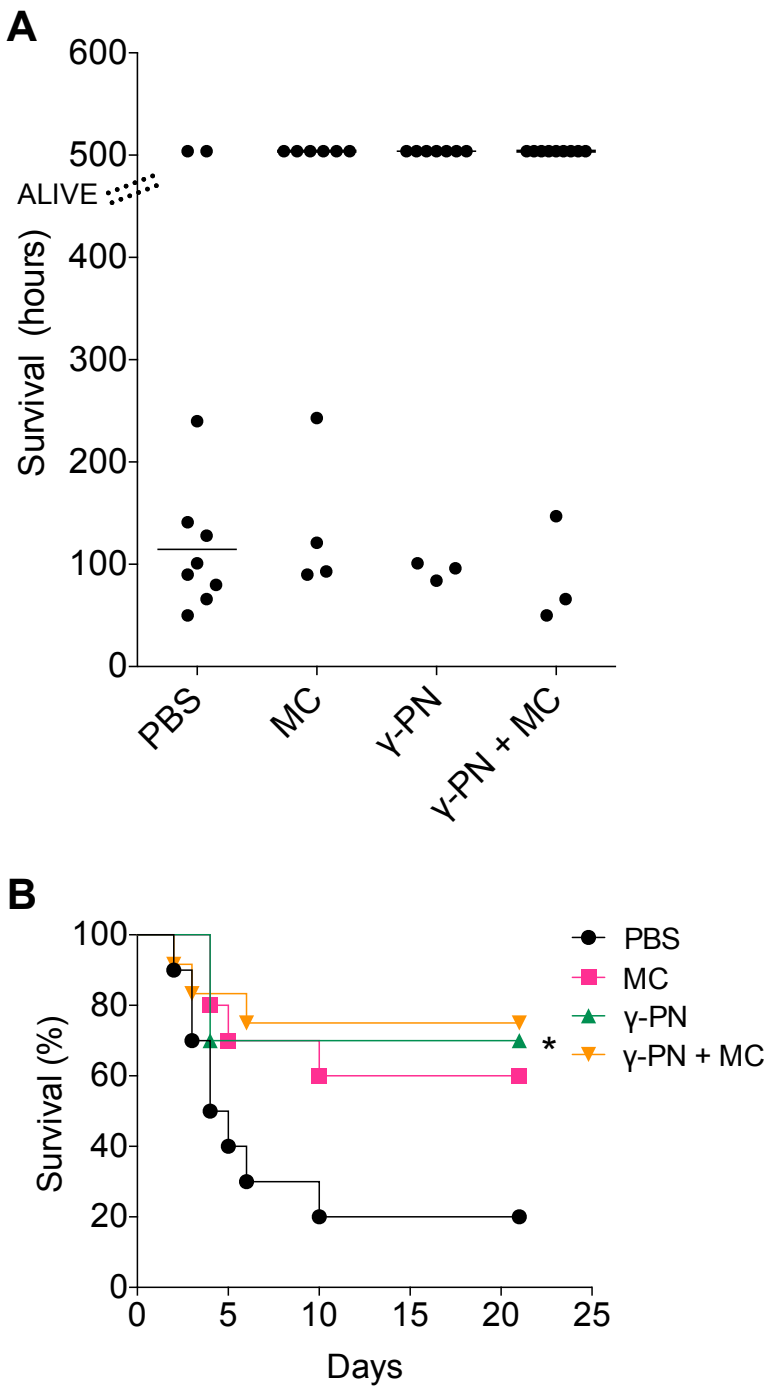


Figure 4.6: Intranasal vaccination with γ-PN + MC does not alter γ-PN vaccine efficacy. Mice (n = 10) were immunized IN with 2 doses of γ-PN or γ-PN + MC, using 20 µg/mouse of MC (Section 2.12-13). Control groups were treated with PBS or MC. 2 weeks after the second immunisation, mice were challenged IN under anaesthesia with D39 (Section 2.14). Survival time (A) and survival rates (B) are shown for a period of 21 days. Data were analyzed using a Fisher exact test (*, $P < 0.05$) (survival rate).

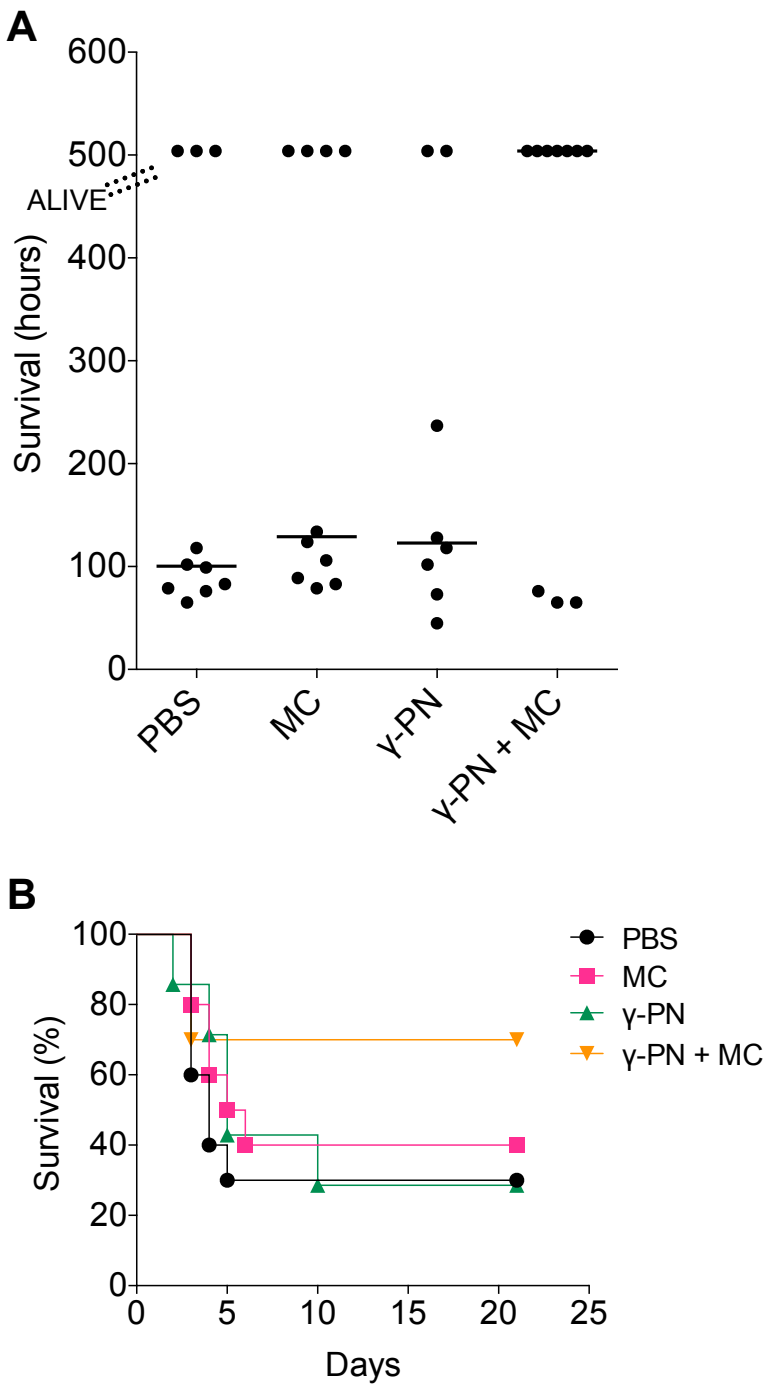


Figure 4.7: Using a reduced dose of MC is not associated with significant enhancement in γ-PN vaccine efficacy. Mice (n = >8 per group) were immunized IN with 2 doses of γ-PN or γ-PN + MC, using 6 µg/mouse of MC. Control groups were treated with PBS or MC (Section 2.12-13). 2 weeks after the second immunisation, mice were challenged IN under anaesthesia with D39 (2.5×10^6 CFU). Survival time (A) and survival rates (B) are shown for a period of 21 days.

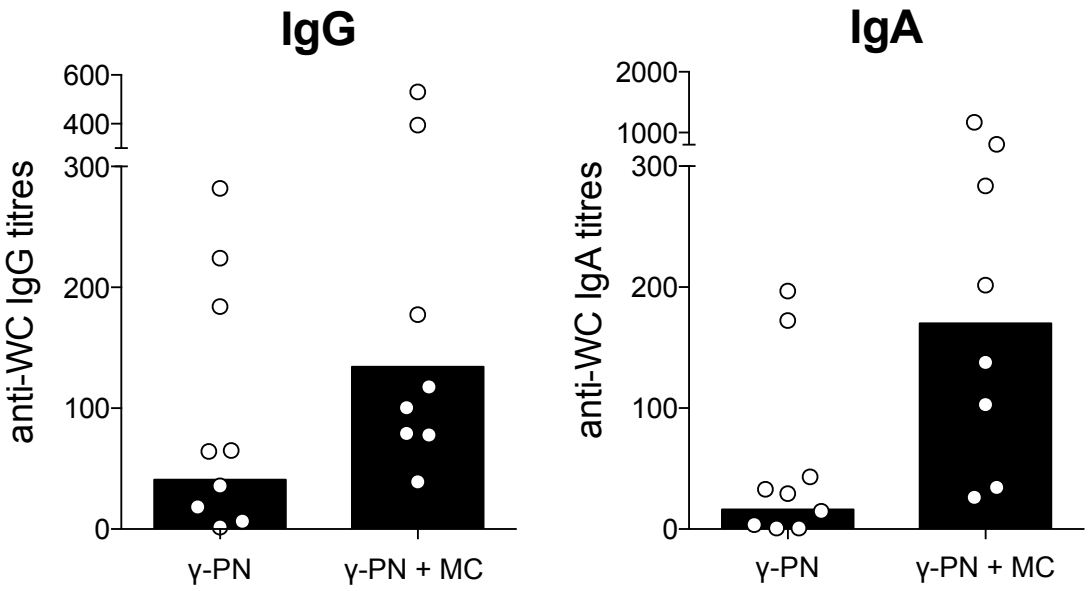


Figure 4.8: Intranasal vaccination with γ-PN + MC does not have a significant impact on γ-PN vaccine immunogenicity. Mice ($n \geq 8$) were immunized IN with 2 doses of γ-PN or γ-PN + MC, using 6 μg/mouse of MC (Section 2.12-13). 2 weeks after the second immunisation, sera were collected and assayed for IgG-specific and IgA-specific antibodies against the whole-cell vaccine strain by ELISA. Geometric mean for antibody titre is shown.

cells specific against the γ-PN vaccine and MalX antigen, which is a prominent antigen in inducing IL-17 (86). Considering that CT has been reported to promote Th17 dominating responses to bystander antigens as well as specifically to the CT B subunit (283, 394), it is reasonable to expect the induction of Th17 memory cells when γ-PN is co-administered with CT as opposed to vaccination with γ-PN alone. However, the lack of protection against colonization when CT is employed as an adjuvant suggests that Th17 memory cells may be involved in other aspects of protection aside from colonization in this model. Th17 cells are well known to enhance the recruitment of macrophages and neutrophils to promote phagocytic activity for bacterial clearance (84, 395). They have also been shown to promote the induction of anti-microbial peptides (including S100 proteins and β-defensins) (396) and have been implicated in up-regulation of polymeric Ig receptor for transport of secretory IgA into the lumen (397).

In addition to the induction of Th17 memory cells, analysis of lung tissues revealed that vaccination with γ-PN + CT promotes the development of TRM cells. However, the difference in TRM cells observed between PBS and CT vaccinated groups indicates that the CT treated group induces high levels of non-specific immunity. Nevertheless, the data suggest the possible development of $CD4^+$ TRM when CT is employed as an adjuvant in contrast to vaccination with γ-PN alone. To my knowledge, this is the first experimental evidence for the development of TRM cells during pneumococcal vaccination. Establishment of TRM at the portal of pathogen entry is important to enhance vaccine efficacy by providing rapid immune responses without relying on recruitment of effector memory responses from circulation. Thus, it appears that the presence of CT during immunisation with γ-PN may have induced differentiation of these distinct memory cells. It has been reported that cytokines such as IL-15 and IL-33 are implicated in the development of TRM and maintenance of TRM in specific tissues (206, 398). It has also been shown that TGF-β driven expression of CD103 maintains TRM cells (398). The presence of TRM memory cells in the lungs of γ-PN + CT vaccinated mice may contribute to the observed protection against the lethal pneumococcal challenge. However, further experiments are required to confirm the cytokine profiles and the fate of these cells in the context of live pneumococcal infection.

In addition to the induction of cellular immune responses, the data illustrate the significant enhancement of pneumococcal-specific antibody titres in mice immunized with γ-PN + CT in comparison to vaccination with γ-PN alone. This is expected as CT has been reported to

promote strong IgG and IgA responses to co-administered antigens (278, 284, 399). Thus, enhanced humoral responses, in conjunction with Th17-cells and possible development of TRM cells, could be responsible for the enhanced protection in γ-PN + CT vaccinated mice. The lack of protection against nasopharyngeal carriage observed in these mice is contrary to other reports regarding CT adjuvanted pneumococcal whole-cell vaccines (82, 133). However, the inability to clear colonizing bacteria could conceivably be beneficial considering the concerns related to vaccines targeting nasopharyngeal carriage as clearance may promote the replacement of colonized pneumococci with other bacteria such as *S. aureus* (374).

Considering the safety concerns related to clinical use of CT (378), chitosan was also evaluated as a mucosal adjuvant with the γ-PN vaccine. Chitosan is considered a safe and well-tolerated intranasal adjuvant (400), as it has the ability to improve antigen delivery through opening of epithelial tight junctions (376, 385, 386). In addition, the muco-adhesive property of chitosan has been reported to prolong retention time at the nasal mucosa (401). The addition of chitosan to whole-cell pneumococcal vaccines has not been investigated previously. In this study, MC was used, which is a form of chitosan that is coupled with mannose groups as a means to promote mannose receptor-mediated endocytosis (402). This is expected to facilitate the uptake of pneumococcal whole-cells for antigen processing and presentation by cells that express mannose receptors such as dendritic cells and macrophages (403, 404). Interestingly, the data show that co-administration of MC with γ-PN did not appear to significantly enhance the immunogenicity or the protective efficacy of the γ-PN vaccine against lethal pneumococcal challenge. Although mice vaccinated with γ-PN and the lower dose of MC showed higher survival rates when challenged with the substantially increased dose of D39, these observations did not reach statistical significance. Moreover, the addition of MC with γ-PN did not induce significantly higher levels of IgG and IgA in comparison to mice vaccinated with γ-PN alone, despite the apparent elevated levels. Thus, the data indicate that MC at a dose of 6 μg/mouse has limited potential as an adjuvant with the γ-PN vaccine. Analysis of two different concentrations of MC illustrated the association between high concentrations and non-specific immune responses. Therefore, it is possible that the concentration of MC was not optimal to evaluate the full potential of MC as an adjuvant, which could be addressed in future studies. In addition, due to small samples sizes, experiments may be worth repeating. Furthermore, factors associated with different forms of chitosan have also been considered to influence the effectiveness of chitosan as an adjuvant,

such as the degree of deacetlylation, molecular weight, pH and the density of charge. To overcome some of these factors, alternative derivatives of chitosan have been synthesized such as thiolated chitosan, N-trimethyl chitosan, mono-N-carboxymethyl chitosan and Poly (ethylene glycol)-g-chitosan (405). In particular, N-trimethyl chitosan has been used to coat whole inactivated influenza virions and was reported to improve immunogenicity and antigen delivery (406). Thus, future studies could evaluate the effectiveness of alternative chitosan derivatives with the γ-PN vaccine.

Overall, the work described in this chapter demonstrates that the immunogenicity of the γ-PN vaccine can be greatly influenced by the presence of an appropriate adjuvant. The data show that CT can effectively modulate the type of immune response induced by γ-PN from Th17-independent immunity and innate IL-17 responses to Th17-dependent immunity and possible development of TRM cells. The development of Th17 responses, in particular, is highly desirable for the induction of long-term memory responses against *S. pneumoniae.* Thus, a Th17 polarizing adjuvant may be most appropriate to enhance the immunogenicity of the γ-PN vaccine. However, knowing the safety concerns associated with CT, future studies should evaluate the role of other mucosal adjuvants.

CHAPTER 5

Intranasal co-vaccination with γ-PN and γ-FLU

5.1 Introduction

Secondary bacterial infection by *S. pneumoniae* following an initial influenza infection is specifically associated with high mortality rates, particularly during influenza pandemics. Lethal synergism of these two pathogens may result from virus-associated bacterial pneumonia in two distinct scenarios: i) infection of a non-pneumococcal carrier with influenza followed by exposure to *S. pneumoniae*; and ii) influenza infection of an asymptomatic carrier of *S. pneumoniae*. In both cases, influenza shapes the respiratory environment to influence or enhance the host's susceptibility to pneumococcal infection (359, 369) (Section 1.3.1). Thus, vaccination against influenza and/or *S. pneumoniae* represents an essential control strategy against these two globally important pathogens.

Previous reports have highlighted the positive impact of monovalent vaccination against either pathogen or dual vaccination at separate administration sites on the outcome of co-infection (247-251, 407). However, despite the success observed with single vaccination regimes, lack of effective vaccines that offer broad-spectrum, serotype-independent and heterosubtypic protection against *S. pneumoniae* and influenza, respectively, still remains a significant problem (98, 142). The possibility of combining a whole-cell killed bacterial vaccine and an inactivated whole virus vaccine to overcome the synergism of influenza and *S. pneumoniae* infection has not been addressed previously. This is despite the fact that the concept of 'combination vaccines' has been utilised against multiple pathogens (Section 1.4). One of the major advantages of combined vaccines is their ability to induce protective immunity to several pathogens simultaneously, thereby minimizing the number of injections individuals have to experience. This in turn significantly simplifies routine immunization schedules and compliance (408).

Generally, in combination vaccines, it is important that the immune response induced by each of the vaccine antigens does not inhibit or interfere with the quality of immunity induced by single antigens. However, the type of immune responses induced by viruses and extracellular bacteria differs significantly. Influenza is an intracellular pathogen which promotes the induction of both Th1 and cytotoxic $CD8^+$ T cell responses as well as neutralising antibodies, whereas *S. pneumoniae* is an extracellular pathogen that promotes both Th17 cell responses and opsonic neutralising antibodies (409). A previous study has demonstrated the effectiveness of protecting against influenza and *S. pneumoniae* individual infections by

generating a bivalent vaccine, which contained a replication-incompetent hemagglutinin knock out influenza strain expressing the antigenic region of PspA (254). The possibility of mixing a whole-cell killed bacterial vaccine and an inactivated whole virus vaccine in a single vaccination regime against both of these pathogens has not been explored before.

The effectiveness of using gamma-irradiation for development of a *S. pneumoniae* vaccine (γ-PN) has been previously shown in Chapter 3. In addition to the *S. pneumoniae* vaccine, the advantage of using gamma-irradiation as an inactivation technique for development of an influenza vaccine (γ-FLU) has also been reported (222, 329). Intranasal vaccination with γ-FLU has been shown to induce heterosubtypic protection against lethal challenge with different influenza strains, including avian H5N1 virus. In addition, it has also been demonstrated that γ-FLU may have the potential to serve as an adjuvant, since its addition to a poorly immunogenic gamma-irradiated Semliki Forest virus vaccine (γ-SFV) resulted in an enhanced anti-SFV antibody response (348). In light of these findings, this study investigated whether intranasal co-immunisation with γ-FLU and γ-PN could provide effective protection against each of the individual pathogens, as well protecting against enhanced pathogenesis associated with co-infection, without compromising pathogen-specific immune responses.

5.2 Results

5.2.1 The immunogenicity and protective efficacy of γ-FLU.

To investigate the efficacy of combining γ-FLU with γ-PN, a new batch of gamma-irradiated influenza vaccine was generated as described in Section 2.5 using the influenza strain A/PR8 (H1N1). To ensure that this newly generated vaccine was protective, mice were immunized with γ-FLU or given PBS. 3 weeks later, mice were challenged with a lethal dose of A/PR8 (H1N1)(the same strain as the γ-FLU vaccine strain; 'homotypic challenge') or A/PC (H3N2) (a different strain to the vaccine; 'heterosubtypic challenge'). Mice vaccinated with γ-FLU elicited significant protection against homotypic challenge as indicated by survival rates (Figure 5.1A) and weight loss (Figure 5.1B) in comparison to the PBS control (euthanasia when 20% weight loss). Similarly, vaccinated mice were also able to elicit significant protection against heterosubtypic challenge as shown with survival rates (Figure 5.2A). Considering the T cell based protection against heterosubtypic influenza challenge (223), these mice appeared to succumb to infection and started to lose body weight prior to recovery at day 8 and returned to original weight by the end of the monitoring period (Figure 5.2B).

During influenza infection, antibody responses generated against surface proteins (HA and NA) are important for virus neutralization (179). In addition, cytotoxic T cell responses are essential for killing viral-infected cells and mediating cross-protection against multiple subtypes of influenza (188). Therefore to confirm whether the γ-FLU vaccine was able to induce effective immune responses, firstly sera were collected from vaccinated mice and analyzed for influenza-specific antibody levels by ELISA. As shown in Figure 5.3, γ-FLU vaccinated mice induced high levels of influenza-specific IgG. To analyze the ability of the γ-FLU vaccine to elicit effective cytotoxic T cell responses, mice were injected intravenously with either live A/PR8, γ-FLU or given PBS (Control) and analyzed for their ability to induce cytotoxic T cell responses using an *in vivo* cytotoxic T cell assay. As shown in Figure 5.4, there were comparable percentages for both the NPP-pulsed and unpulsed cells in the spleens from control mice, indicating the absence of cytotoxic T cell responses in these mice. In contrast, mice injected with either live A/PR8 or γ-FLU had significantly lower percentages (~5% for A/PR8, ~13% for γ-FLU) of the NPP pulsed cell population relative to the proportion of unpulsed cells, indicating the ability of these mice to induce effective cytotoxic T cell responses against target splenocytes presenting influenza-specific peptide in the context of MHC-I. Overall these data confirm the ability of the γ-FLU vaccine to mediate cross protection against different influenza subtypes and the ability to induce effective immune responses.

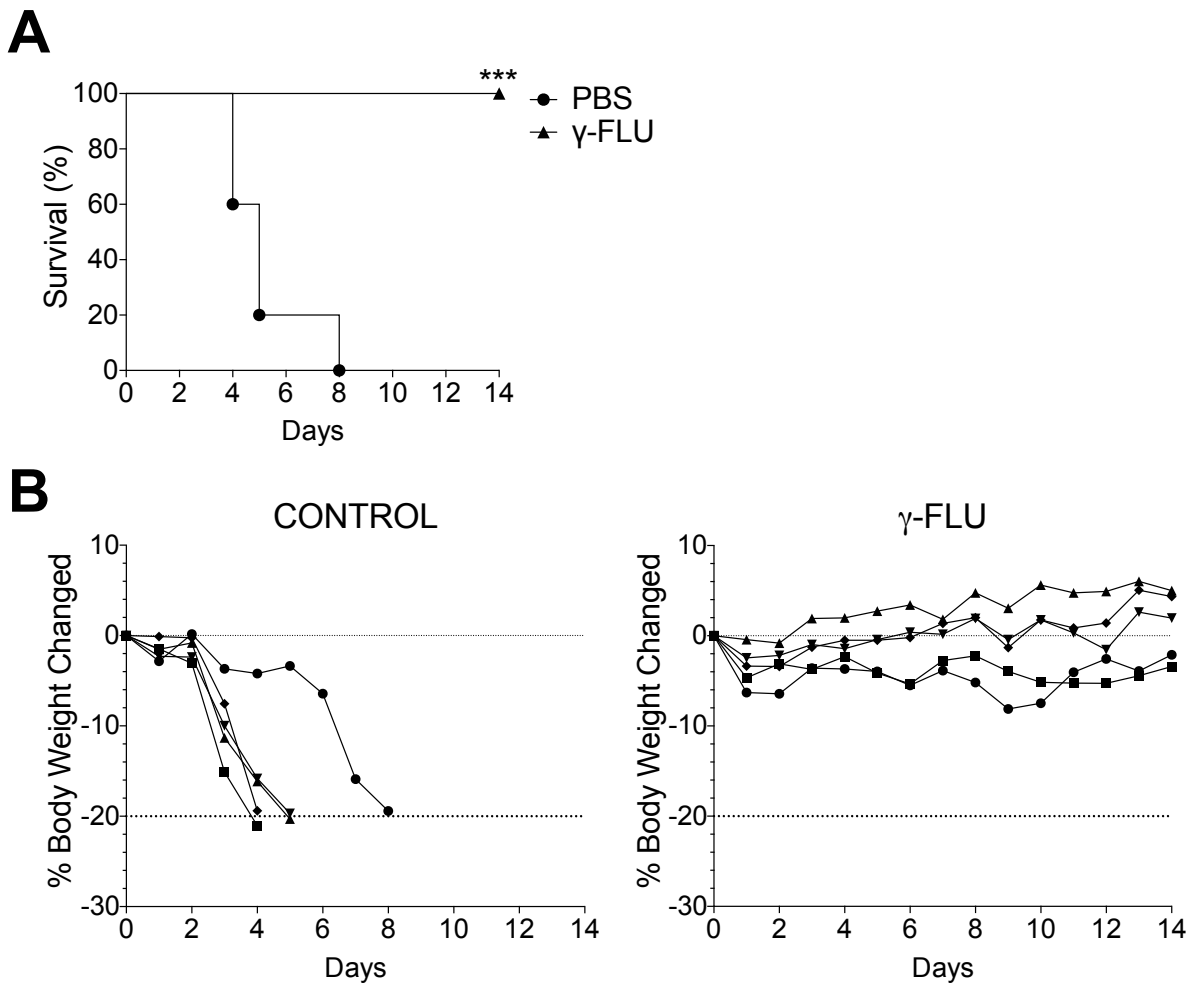


Figure 5.1: Intranasal vaccination with γ-FLU provides homotypic protection against A/PR8 challenge. Mice (n = 5) were immunized IN with γ-FLU or given PBS (control) (Section 2.12). 3 weeks later, mice were challenged IN under anaesthesia with A/PR8 (~200 $TCID_{50}$) (Section 2.14). (A) Survival rates and (B) percentage weight loss is shown for a period of 14 days. The dotted line represents 20% weight loss (trigger for euthanasia). Data were analyzed using a Fisher exact test (***, $P < 0.001$).

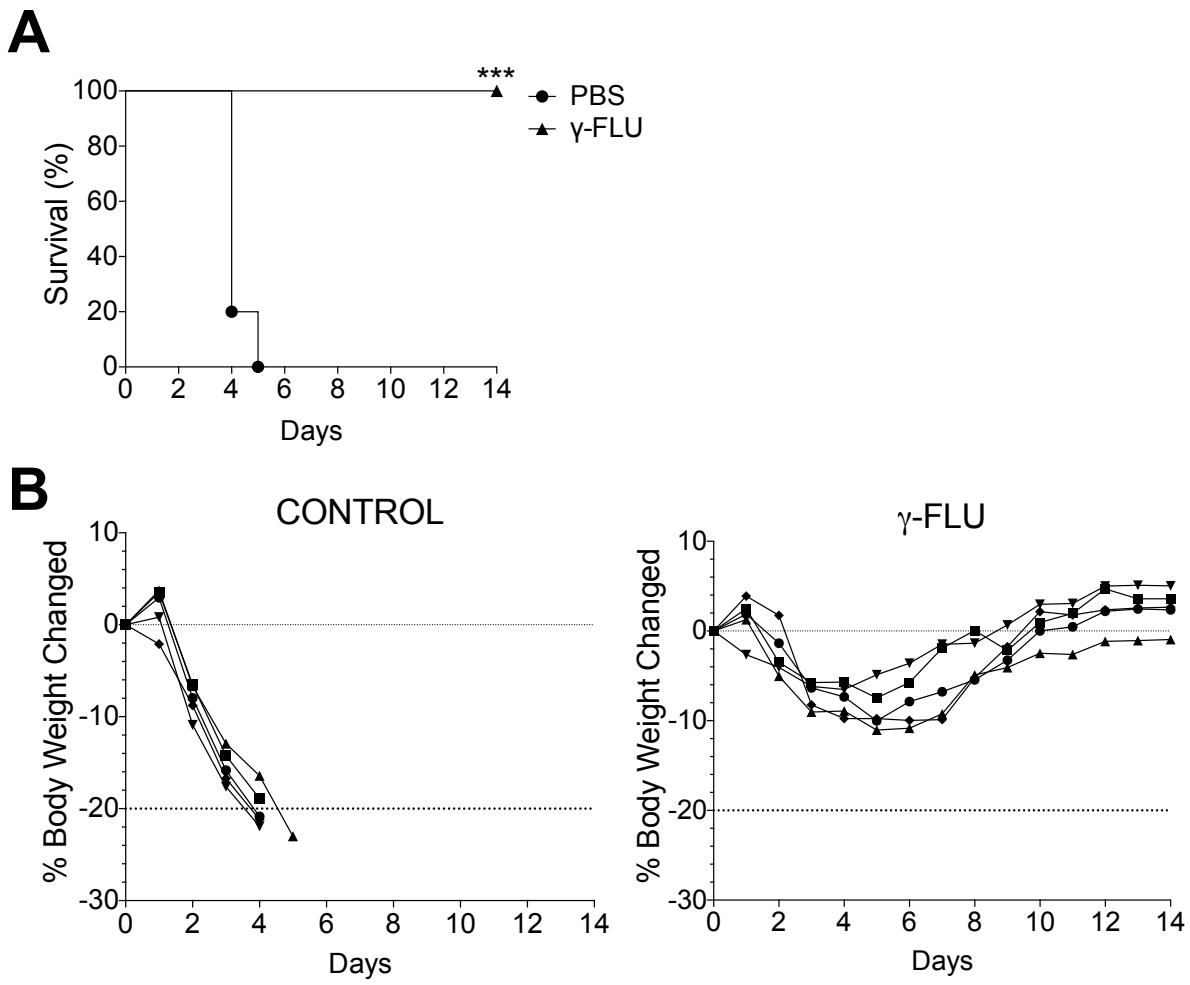


Figure 5.2: Intranasal vaccination with γ-FLU provides heterosubtyic protection against A/PC challenge. Mice (n = 5) were immunized IN with γ-FLU or given PBS (control) (Section 2.12). 3 weeks later, mice were challenged IN under anaesthesia with A/PC (9.6×10^3 $TCID_{50}$) (Section 2.14). (A) Survival rates and (B) percentage weight loss is shown for a period of 21 days. The dotted line represents 20% weight loss (trigger for euthanasia). Data were analyzed using a Fisher exact test (***, $P < 0.001$).

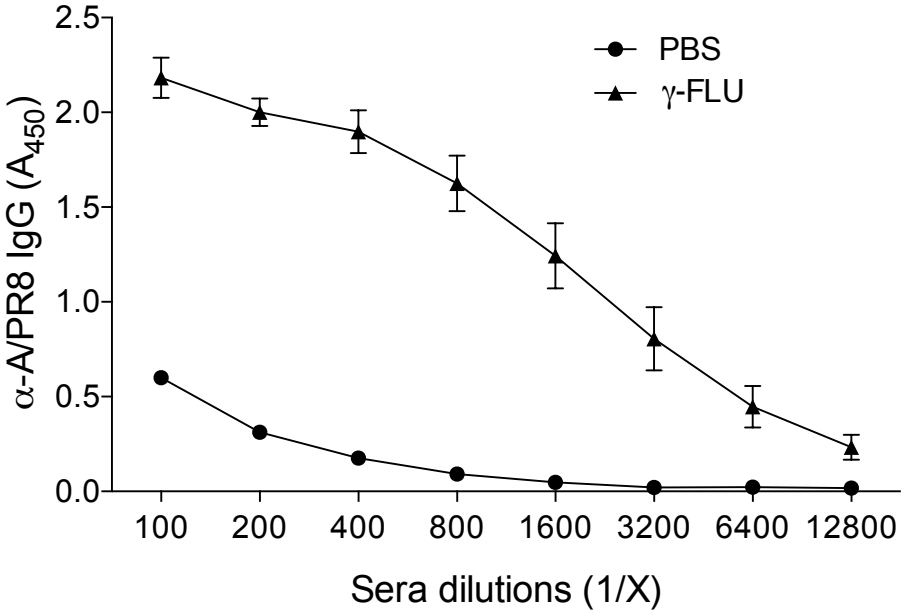


Figure 5.3: γ-FLU induces effective antibody responses. Mice (n = 5) were immunized IN with γ-FLU or given PBS (control) (Section 2.12). 3 weeks later, sera were collected and analyzed for influenza-specific IgG titres by ELISA. Absorbance levels are shown.

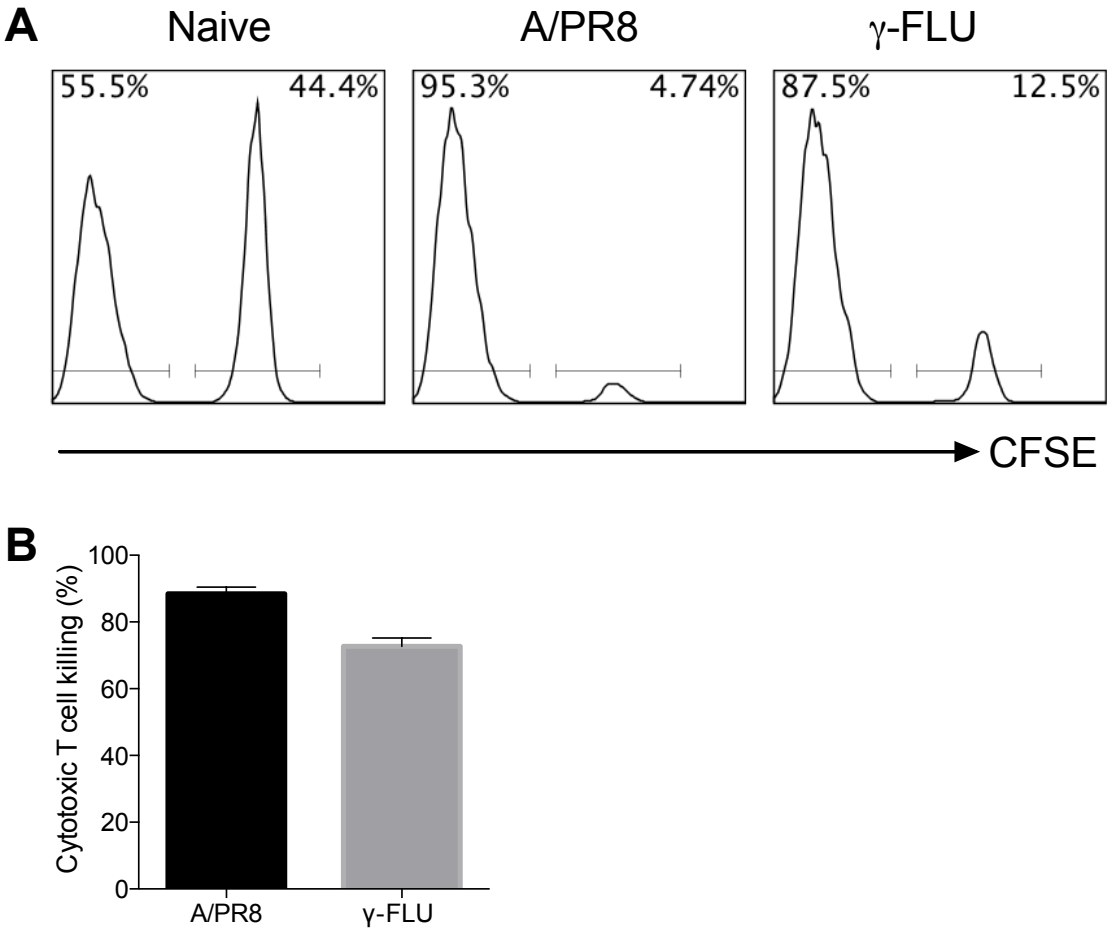


Figure 5.4: γ-FLU induces effective cytotoxic T cell responses. Mice were immunized intravenously (IV) with live A/PR8 (~1 × 10^7 $TCID_{50}$), γ-FLU (1 × 10^8 $TCID_{50}$) or given PBS. 1 week post immunization, target splenocytes were labelled and injected as described in Section 2.21. 18 hours post injection of target splenocytes, the spleens were harvested and analyzed for the percentage of labelled target cells ($CFSE^+$, 'pulsed') by flow cytometry. (A) Representative histogram showing the percentage of pulsed cells ($CFSE^+$) and unpulsed cells ($CFSE^-$). (B) Graph showing the percentage of cytotoxic T cell killing in mice previously immunized with either A/PR8 or γ-FLU. Results are representative of 2 independent experiments.

5.2.2 Co-immunisation enhances the protective efficacy of the γ-PN vaccine.

To determine whether administering γ-PN + γ-FLU as a combined vaccine modulates the protective efficacy of the whole pneumococcal vaccine, mice were immunized intranasally with γ-FLU, γ-PN or co-immunized with a mixture of γ-PN and γ-FLU (designated γ-PN + γ-FLU). Two weeks after the second immunisation dose, mice were challenged intranasally with D39 and monitored for survival. As shown in Figure 5.5A & 5.5B, immunisation with γ-PN either alone or co-administered with γ-FLU resulted in significant protection against lethal D39 challenge compared to control mice (survival: $P = 0.0349$ (γ-PN), $P = 0.0089$ (γ-PN + γ-FLU)). Co-vaccinated animals showed a slightly higher rate of survival compared to those vaccinated with γ-PN alone. As expected, γ-FLU immunization alone did not protect mice from lethal pneumococcal challenge (Figure 5.5A & 5.5B). Thus, co-immunisation of γ-PN + γ-FLU vaccines does not adversely affect the protective efficacy of γ-PN vaccination in a model of lethal pneumococcal sepsis (D39 challenge). To further investigate the efficacy of the combination vaccine, mice were challenged with EF3030 in the colonization-induced pneumonia model. Strikingly, mice co-immunized with γ-PN + γ-FLU were the only group to show a significant reduction in bacterial counts in both the nasopharynx and the lungs relative to the PBS control ($P = 0.0436$ (lungs), $P = 0.00436$ (nasopharynx)) (Figure 5.6A). Co-immunized mice were also the only group to show significantly increased survival rates compared to the PBS control ($P = 0.0010$) (Figure 5.6B). Overall, these data demonstrate that co-immunisation with γ-PN + γ-FLU does not compromise but rather enhances the protective efficacy of the γ-PN vaccine.

As observed in the colonization-induced pneumonia model, vaccination with the combination vaccine resulted in a significant reduction in nasopharyngeal counts. Therefore, to determine whether the combination vaccine mediates protection against colonization only, immunized mice were colonized with EF3030 (colonization model) and bacterial counts were analyzed in the nasopharynx. Vaccination with γ-PN alone did not have an impact on the levels of colonization as bacterial counts were similar to that observed in control mice (PBS treated) (Figure 5.7). However, co-immunized mice showed significantly reduced bacterial loads in the nasopharynx relative to the PBS and γ-PN vaccinated groups. Overall, the results confirm that co-immunisation with γ-PN + γ-FLU mediates protection against pneumococcal colonization.

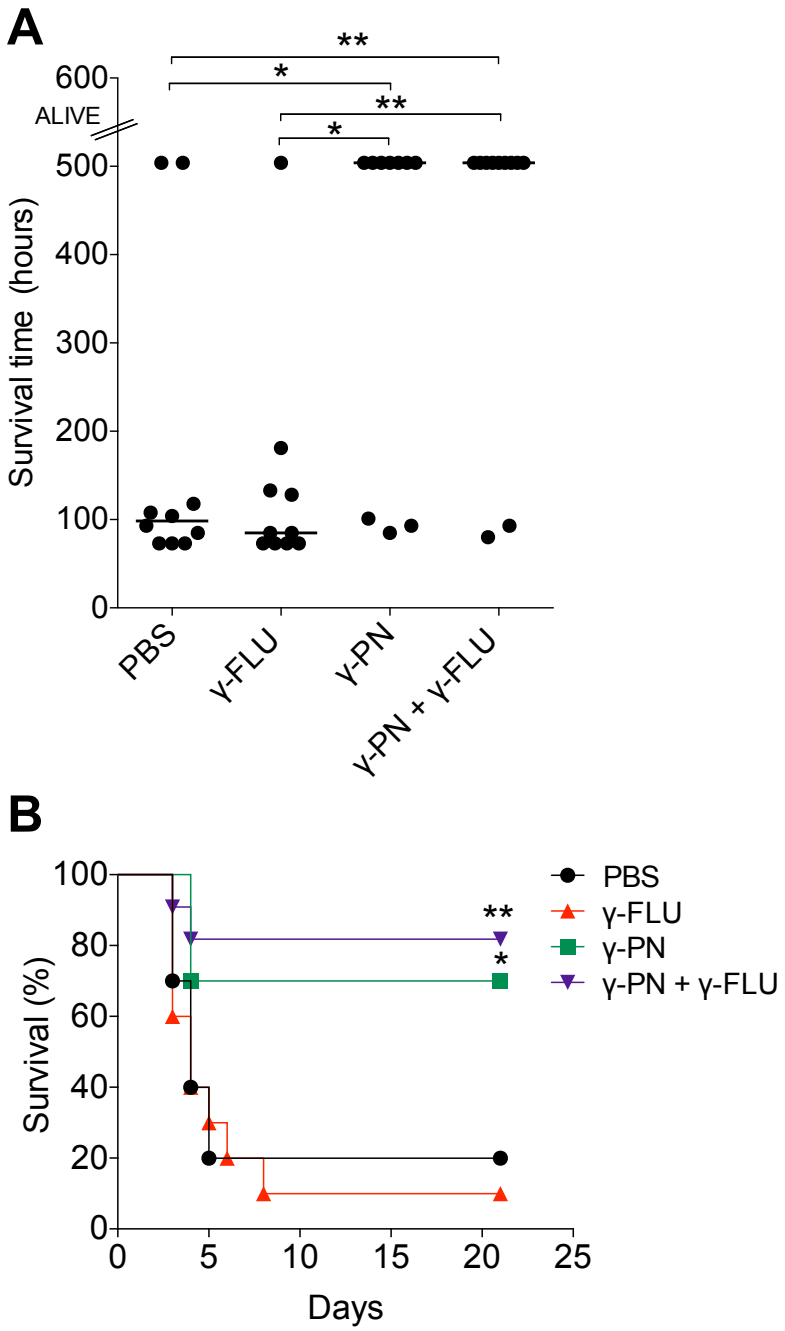


Figure 5.5: Co-vaccination with γ-PN + γ-FLU does not affect the protective efficacy of γ-PN vaccine against live D39 challenge. Mice (n = 20, data pooled from 2 independent experiments of n = 10) were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were challenged IN under anaesthesia with D39 (Section 2.14) and (A) survival time and (B) overall survival rates are shown for a period of 21 days. Data were analyzed using One-way ANOVA (survival time) and Fisher exact test (survival rates) (*, $P < 0.05$; **, $P < 0.01$).

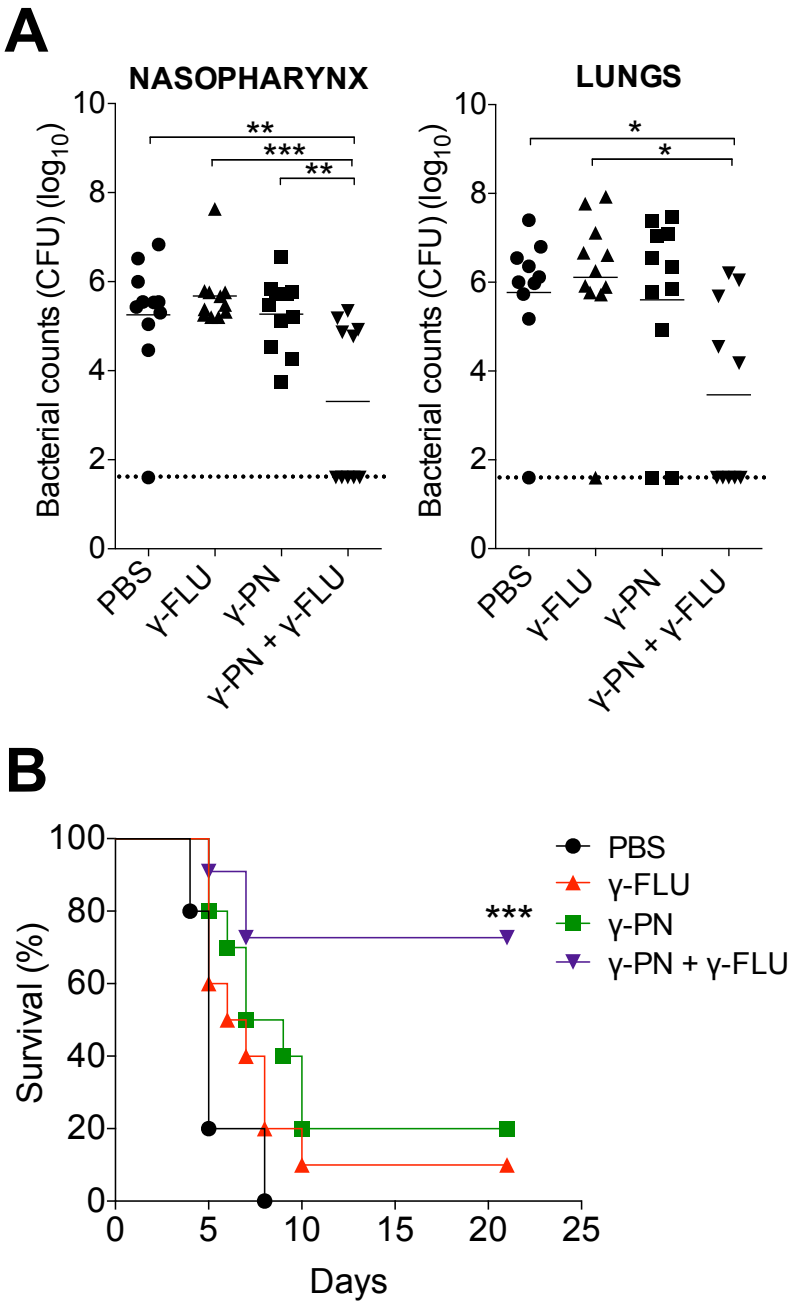


Figure 5.6: Co-vaccination with γ-PN + γ-FLU enhances the vaccine efficacy of γ-PN. Mice (n = 10) were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were colonized IN with EF3030 and 4 days later were anaesthetized and given PBS IN (colonization-induced pneumonia model) (Section 2.14). (A) Bacterial counts in the lungs and nasopharynx were assessed at day 8 post challenge and (B) Survival rates are shown for a period of 21 days after challenge with PBS IN. Data were analyzed using one-way ANOVA (bacterial loads) and Fisher exact test (survival rates) (*, $P < 0.05$; ** $P, < 0.01$; *** $P, < 0.001$).

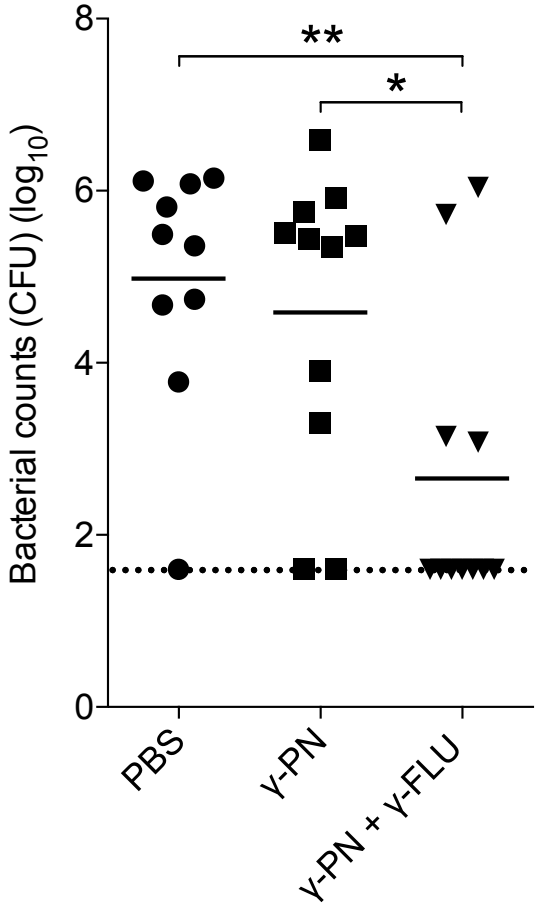


Figure 5.7: Co-vaccination with γ-PN + γ-FLU mediates protection against nasopharyngeal colonization. Mice (n = 10) were immunized IN with 2 doses of γ-PN or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were colonized IN with EF3030 (colonization model) (Section 2.14) and bacterial counts in the nasopharynx were assessed at day 7 post challenge. Data were analyzed using one-way ANOVA (*, $P < 0.05$; ** P, < 0.01).

5.2.3 Cellular immunity plays a major role in the observed enhanced protection elicited by co-immunisation.

Humoral immunity is an important defence mechanism against *S. pneumoniae* infection. Antibodies against certain cell surface proteins play important roles in complement-mediated opsonisation to facilitate bacterial clearance (1). Thus, to assess how γ-PN + γ-FLU co-immunisation shapes pneumococcal-specific antibody responses, sera were collected from immunized or co-immunized mice and analyzed for pneumococcal-specific IgG and IgA titres by ELISA. As shown in Figure 5.8, co-immunisation enhanced pneumococcal-specific IgG and IgA titres relative to individual immunisation with γ-PN. Therefore, these data indicate that γ-FLU displays adjuvant activity and suggests that augmented antibody responses may be one of the possible mechanisms responsible for the enhanced vaccine efficacy following pneumococcal challenge.

To determine whether the combination vaccine modulated the $CD4^+$ cellular immune response, following immunisation, mice were depleted of $CD4^+$ cells using anti-CD4 depleting antibodies prior to and during challenge with EF3030 in the colonization-induced pneumonia model. Successful depletion (by ~99.5%) of $CD4^+$ cells from lung and spleen was confirmed using flow cytometry (Figure 5.9A&B). The data indicate that co-immunized mice depleted of $CD4^+$ cells were no longer protected against pneumococcal disease, with survival rates of co-vaccinated and unvaccinated (PBS) animals being equivalent (Fig 5.9C). In contrast, co-immunized mice given isotype control antibodies still exhibited significant levels of protection against pneumococcal challenge (P = 0.0426) (Figure 5.9C). Additionally, vaccination with the γ-PN vaccine alone did not provide significant protection in mice treated with either an isotype control or anti-CD4 antibodies. Taken together, these data indicate that vaccination with γ-PN + γ-FLU elicits enhanced protection against pneumonia that is dependent on $CD4^+$ cellular immune responses.

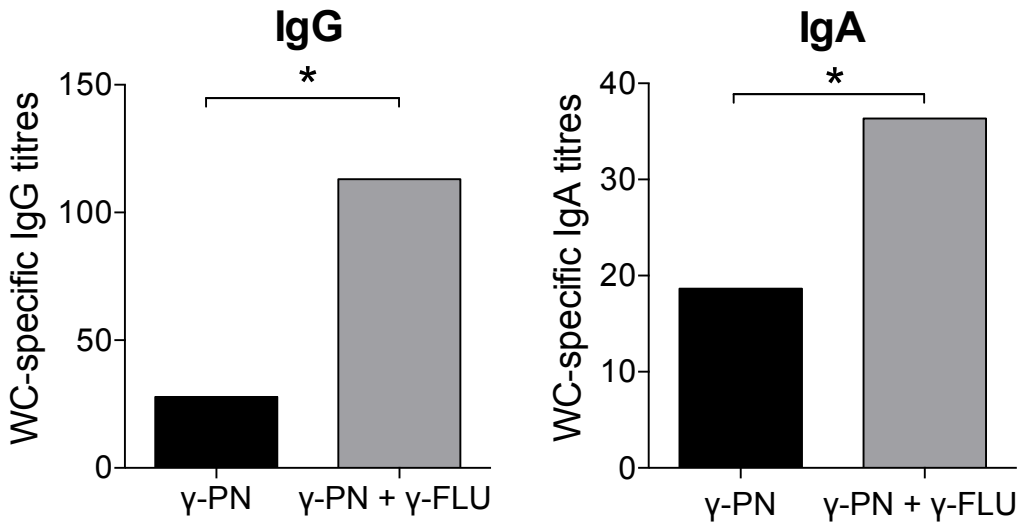


Figure 5.8: Co-vaccination with γ-PN + γ-FLU enhances pneumococcal-specific antibody titres. Mice (n = 8) were immunized IN with 2 doses of γ-PN, or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, sera were collected and analyzed for pneumococcal-specific IgG or IgA titres by ELISA. Geometric mean for antibody titre is shown. Data were analyzed using an un-paired Student's *t*-test (*, $P < 0.05$).

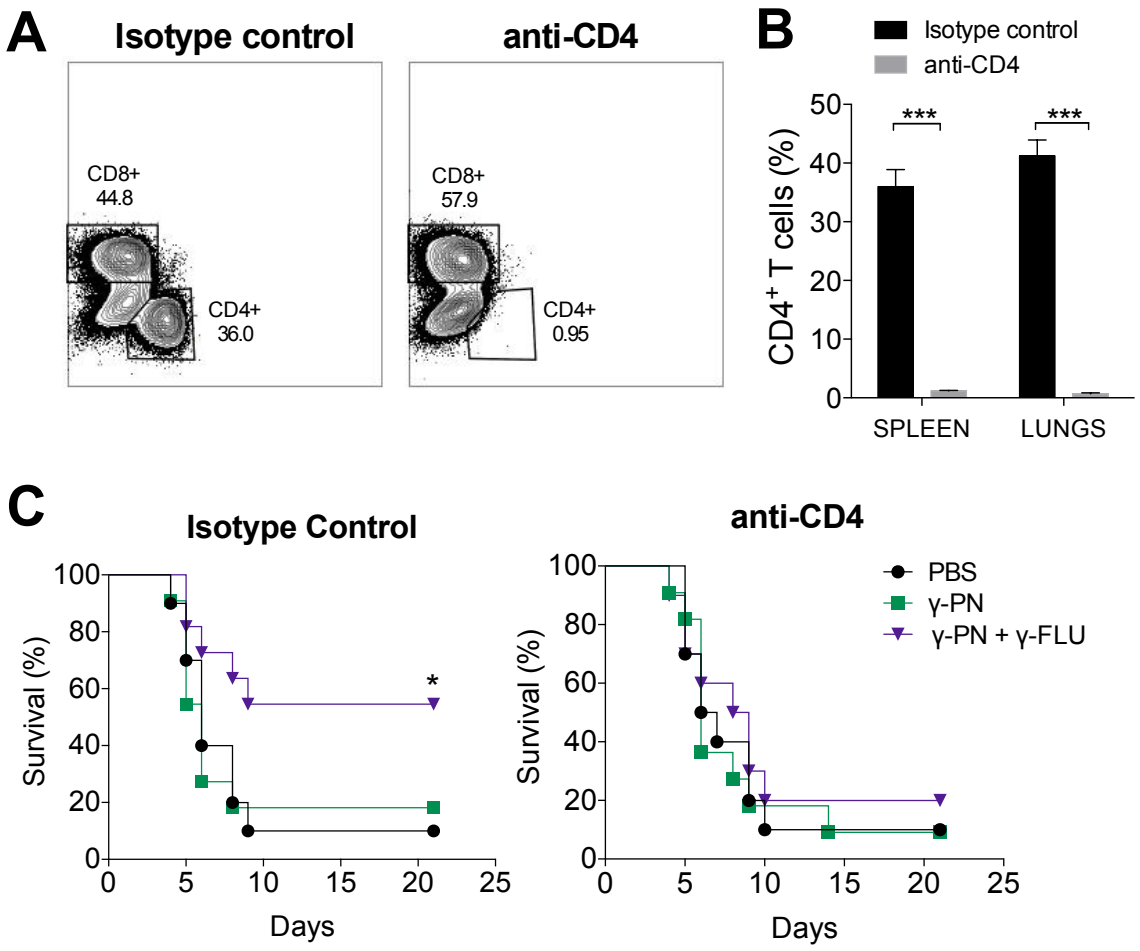


Figure 5.9: The protective efficacy of the combination vaccine is dependent on CD4$^+$ responses. Mice (n = 10) were immunized IN with 2 doses of γ-PN, or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were injected with anti-CD4 (150 μg) or isotype control (anti-IgG2b) at day -2, -1, 1 and 4 post challenge with EF3030 in the colonization-induced pneumonia model (Section 2.14). Flow cytometry was performed to determine the percentage of CD4$^+$ T cells in the spleen and lungs 24 hours after administration of anti-CD4 or isotype control antibodies. (A) Representative FACS plot and (B) histogram showing the percentage of CD4$^+$ T cells in the lungs and spleens for mice administered either anti-CD4 or isotype control (anti-IgG2b) antibodies. (C) Survival rates are shown for a period of 21 days after challenge with PBS IN. Data were analyzed using an un-paired Student's *t*-test (CD4$^+$ T cell percentage) (***, $P < 0.001$) or a Fisher exact test (survival) (*, $P < 0.05$).

5.2.4 Co-immunisation enhances pneumococcal-specific memory Th17 and Th1 cell responses.

As previously shown in Chapter 3, intranasal vaccination with the γ-PN vaccine promotes γδT17 cells not Th17 cells. To explore the nature of the $CD4^+$ dependent immunity associated with enhanced protection induced by co-immunisation with γ-PN + γ-FLU, the type of antigen-specific T cell memory responses that are elicited after *ex vivo* stimulation with the γ-PN vaccine were next investigated. As shown in Figure 5.10, there was a significant increase in the total number of $CD4^+$ effector cells and Th17 cells in the lungs of γ-PN vaccinated mice relative to the unvaccinated group (Figure 5.10A). This was also associated with significant levels of IL-17 in the culture supernatants (Figure 5.10C). Importantly, co-immunisation resulted in a further elevation in the number of $CD4^+$ effector cells, Th17 cells and Th1 cells in the lung suspensions and IL-17 levels in the supernatants relative to both the PBS control and the γ-PN vaccinated group (Figure 5.10A&C). Moreover, antigen-specific Th17 cells were only detected in spleens of co-immunized animals relative to the PBS control and this also corresponded with significant levels of IL-17 detected in culture supernatants. No changes in IFN-γ levels in supernatants from lung or spleen post γ-PN vaccine antigen stimulation were detected. Similarly, no changes in $CD8^+$ T cell populations were detected (Figure 5.10B). Overall, these data indicate that co-immunisation with γ-PN + γ-FLU enhances $CD4^+$ effector cell generation, including the number of pneumococcal-specific memory Th1 and Th17 cells relative to γ-PN administration alone. This suggests that these T cell memory populations may contribute to the enhanced efficacy previously observed by use of the combination vaccine.

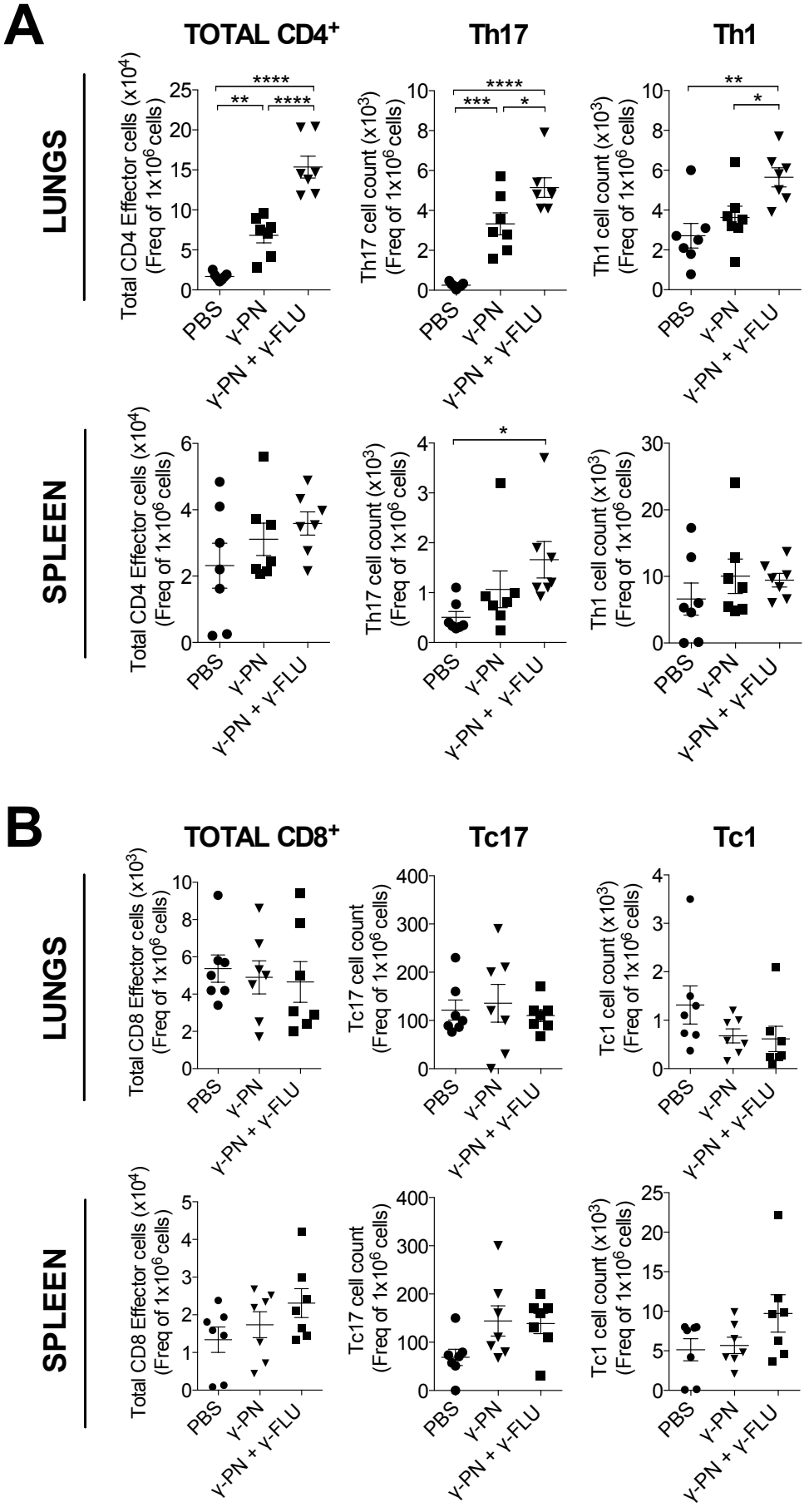
5.2.5 Co-immunisation promotes tissue-resident memory cell development.

Recent reports have highlighted that TRM cells are pivotal in providing immediate protection in the defence against respiratory pathogens such as influenza (209, 410, 411). As previously shown in Chapter 4, immunization with γ-PN + CT appears to promote $CD4^+$ TRM cell development compared to γ-PN alone. Therefore, it was of interest to consider whether the $CD4^+$ dependent immunity observed with co-immunisation is associated with the development of $CD4^+$ TRM cells, especially in light of the ability of influenza viruses to induce TRM. Thus, following immunization, the numbers of lung tissue-resident $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry. TRM cells were gated using the markers $CD44^+$ and $CD103^+$. These cells were previously checked to express CD11a, a common

TRM marker (207, 210). Flow cytometry revealed a significant enhancement in both $CD4^+$ and $CD8^+$ TRM cell populations in the lungs from co-immunized mice relative to the γ-PN vaccinated group and unvaccinated (PBS) controls (Figure 5.11). There was also a significant increase in the number of $CD4^+$ TRM in lungs from the γ-FLU vaccinated group relative to the PBS controls ($P = 0.0256$), and an elevated number of $CD8^+$ TRM cells, but this did not reach statistical significance. As previously reported in Chapter 3, there were no significant alterations in level of $CD4^+$ and $CD8^+$ TRM cell populations from mice immunized with γ-PN alone. The data indicate that co-immunisation with γ-PN + γ-FLU, as well as immunisation with γ-FLU alone, promotes TRM cell development. However, despite the apparent elevated levels of $CD4^+$ TRM in the co-immunized group compared to mice immunized with γ-FLU alone, this difference did not reach significance ($P = 0.1304$).

5.2.6 Co-immunisation promotes Th17 and $CD4^+$ TRM IL-17^+ cell responses to live pneumococcal challenge.

The above *ex vivo* analyses demonstrated that co-immunisation enhances T cell immune responses towards the γ-PN vaccine by promoting pneumococcal-specific Th17 and Th1 cells, and the development of TRM cells, although the antigen specificity of TRM cells was not defined in these experiments. Therefore the frequency of T effector cells and TRM cell populations in the context of a live pneumococcal challenge were investigated. Following D39 challenge, co-immunized mice showed a greater frequency of $CD4^+$ T effector cells relative to mice immunized with γ-PN or γ-FLU alone (Figure 5.12A). This elevation was associated with an increase in Th1 cells relative to mice vaccinated with either γ-FLU or γ-PN, and a significant increase in Th17 cells relative to γ-FLU vaccinated and PBS control mice. Similarly, $CD4^+$ TRM, $CD4^+$ TRM IL-17^+ and $CD4^+$ TRM IFN-γ^+ cells were significantly increased in the co-immunized group relative to the PBS control, γ-FLU and γ-PN vaccinated groups (Figure 5.12B). Alterations in $CD4^+$ effector or TRM cell populations in mice immunized with either γ-FLU or γ-PN alone were not detected. There was also no impact on the number of $CD8^+$ TRM cells for any of the groups following D39 challenge (data not shown). These data indicate that co-immunisation promotes Th17, $CD4^+$ TRM IL-17^+ and $CD4^+$ TRM IFN-γ^+ memory cell responses in the lung upon lethal pneumococcal challenge and implicate these subsets could possibly be the cellular responses underlying the enhanced efficacy observed with the combination vaccine.


A
TOTAL CD4+
Th17
Th1
LUNGS
SPLEEN
Total CD4 Effector cells (x10^4) (Freq of 1x10^6 cells)
Th17 cell count (x10^3) (Freq of 1x10^6 cells)
Th1 cell count (x10^3) (Freq of 1x10^6 cells)
PBS
γ-PN
γ-PN + γ-FLU
B
TOTAL CD8+
Tc17
Tc1
Total CD8 Effector cells (x10^3) (Freq of 1x10^6 cells)
Total CD8 Effector cells (x10^4) (Freq of 1x10^6 cells)
Tc17 cell count (Freq of 1x10^6 cells)
Tc1 cell count (x10^3) (Freq of 1x10^6 cells)

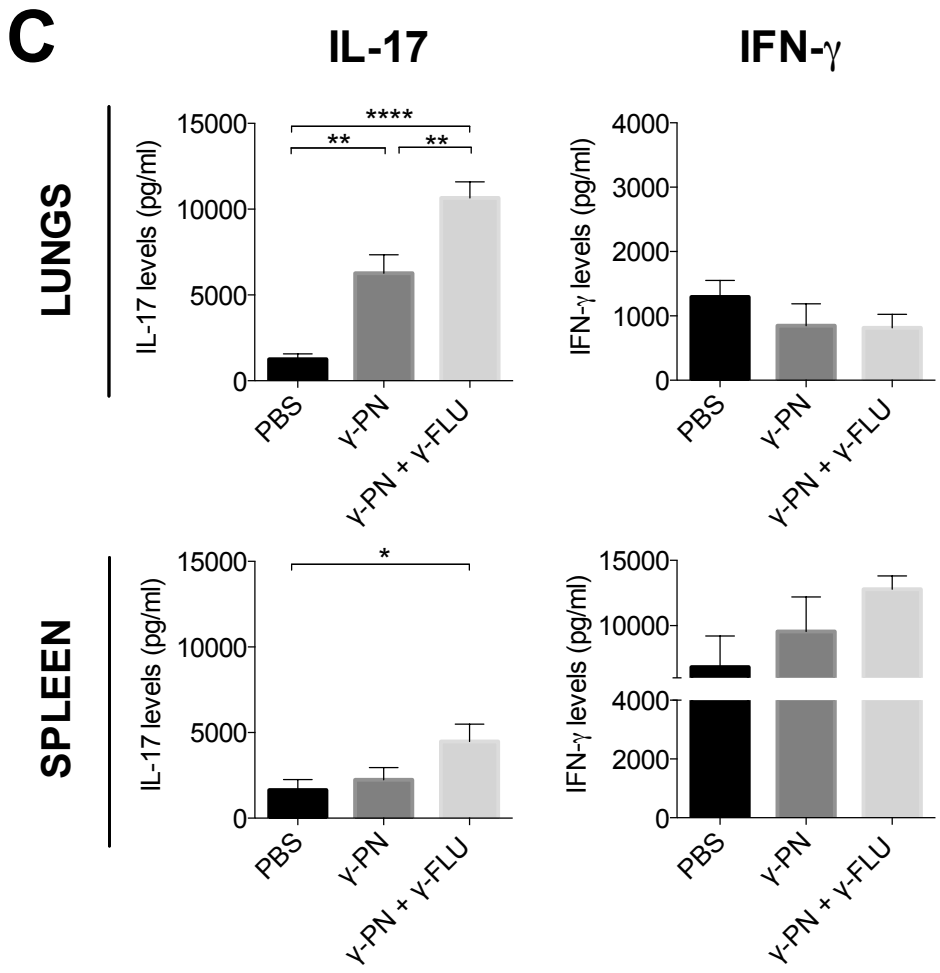


Figure 5.10: Co-immunisation with γ-PN + γ-FLU enhances memory pneumococcal-specific Th17 and Th1 cells. Mice (n = 7) were immunized IN with 2 doses of γ-PN or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, the lungs and spleens were harvested and single cell suspensions were restimulated in culture with the γ-PN vaccine or media alone (negative control) for 72 hours. (A) Frequency of live $CD4^+$ and $CD8^+$ T effector cell subsets in lung and spleen suspensions were determined by flow cytometry: Total $CD4^+$ T effector cells ($CD3^+CD4^+CD44^+$), Th17 ($CD3^+CD4^+CD44^+IL\text{-}17^+$) and Th1 ($CD3^+CD4^+CD44^+IFN\text{-}\gamma^+$) (B) Total $CD8^+$ T effector cells ($CD3^+CD8^+CD44^+$), Th17 ($CD3^+CD8^+CD44^+IL\text{-}17^+$) and Th1 ($CD3^+CD8^+CD44^+IFN\text{-}\gamma^+$) (C) Levels of cytokines (IL-17, IFN-γ) in the supernatant of cultured lymphocytes from the spleens and the lungs. Data were analyzed using One-way ANOVA (*, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

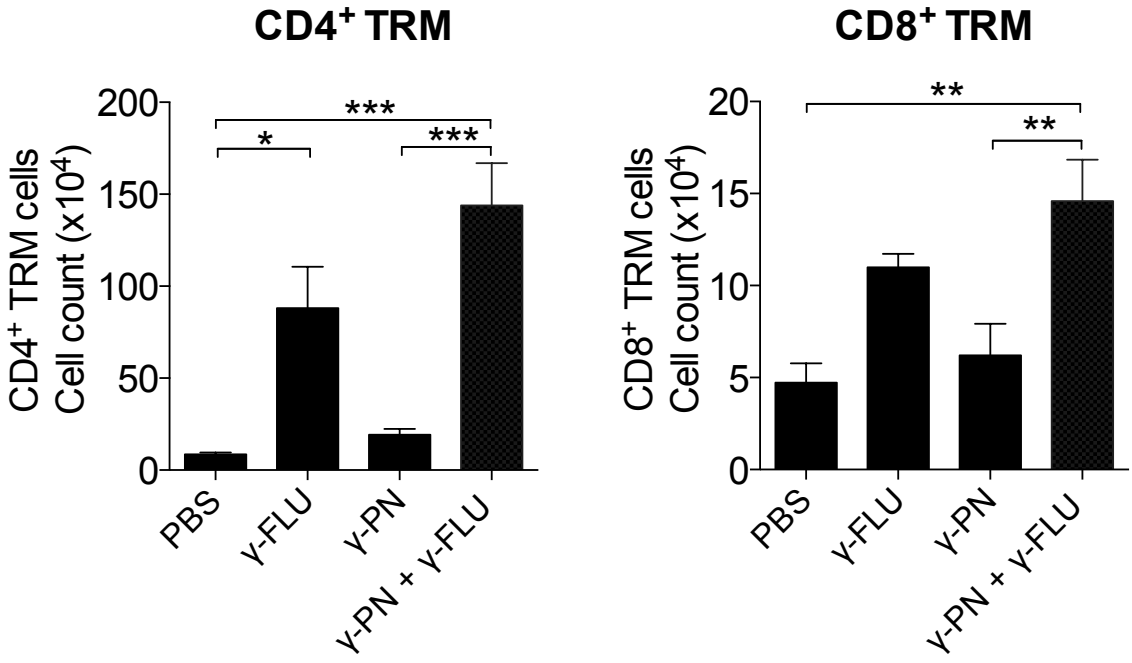


Figure 5.11: Co-vaccination with γ-PN + γ-FLU induces $CD4^+$ and $CD8^+$ TRM cells. Mice (n = 6) were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, the lungs were harvested and analyzed for $CD4^+$ and $CD8^+$ TRM cells by flow cytometry (CD4 TRM: $CD4^+CD8^-CD44^+CD103^+$; CD8 TRM: $CD8^+CD4^-CD44^+CD103^+$). Results are presented as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (One-way ANOVA).

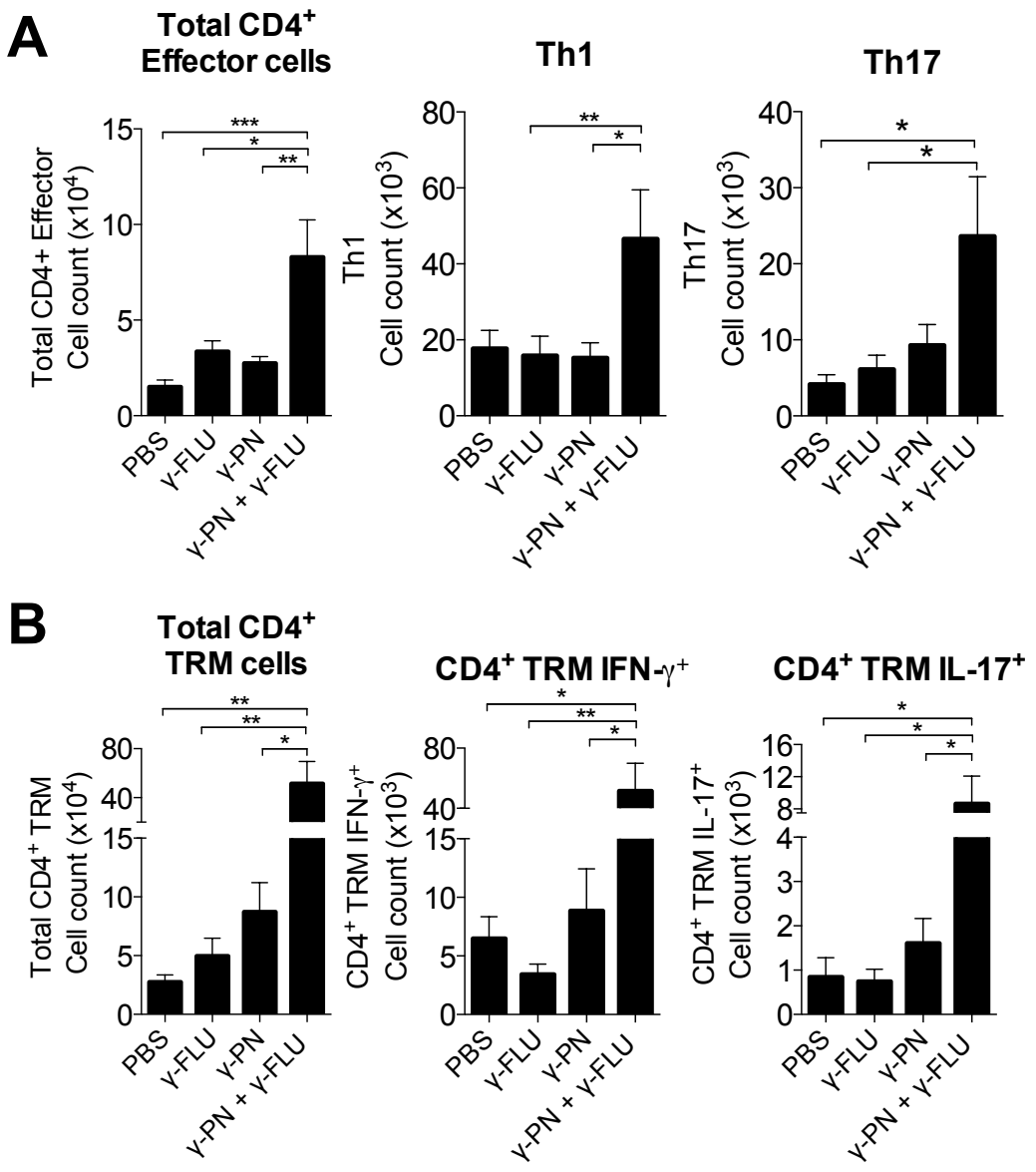


Figure 5.12: Co-vaccination with γ-PN + γ-FLU induces Th17 and CD4$^+$ TRM cells following live D39 challenge. Mice (n = 6) were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were challenged IN with D39 (Section 2.14). 48 hours post challenge, lungs were harvested and analyzed for the total number of T cell populations by flow cytometry. (A) T effector cells: total CD4$^+$ T effector cells (CD3$^+$CD4$^+$CD44$^+$), Th17 (CD3$^+$CD4$^+$CD44$^+$IL-17$^+$), Th1 (CD3$^+$CD4$^+$CD44$^+$IFN-γ$^+$); and (B) TRM cells: Total CD4$^+$ TRM cells (CD3$^+$CD4$^+$CD44$^+$CD103$^+$) and CD4$^+$ TRM IL-17$^+$ cells (CD3$^+$CD4$^+$CD44$^+$CD103$^+$IL-17$^+$) and CD4$^+$ TRM IFN-γ$^+$ cells (CD3$^+$CD4$^+$CD44$^+$CD103$^+$IFN-γ$^+$). Results are presented as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (One-way ANOVA).

5.2.7 Co-immunisation enhances IFN-α levels following live pneumococcal challenge.

As previously reported, γ-FLU was proposed to enhance antibody responses to a co-administered antigen by inducing strong IFN-I responses (348). Therefore, it was of interest to determine whether combination of the two vaccines would alter IFN-I levels. Following D39 challenge, sera were also harvested and analyzed for IFN-α levels by ELISA. As shown in Figure 5.13, co-immunized mice show significantly enhanced levels of serum IFN-α relative to the PBS controls. These data suggest that IFN-α could possibly be a mediator involved in the enhanced efficacy of the combination vaccine.

5.2.8 Co-immunisation does not compromise the protective efficacy of γ-FLU.

An important caveat for combining a whole bacterial (γ-PN) vaccine with an influenza (γ-FLU) vaccine is that dual vaccination should not compromise vaccine efficacy against *S. pneumoniae* or influenza A virus infection. Therefore to assess whether co-vaccination influenced anti-influenza immunity induced by the γ-FLU vaccine, following immunization mice were challenged with a lethal dose of influenza A/PR8 and monitored for survival (euthanasia at 20% weight loss). Figure 5.14 shows that mice immunized with γ-FLU alone or co-immunized with γ-PN + γ-FLU exhibited significant protection against lethal A/PR8 challenge, with both groups showing comparable survival rates ($P = 0.0039$ for both groups) and no body weight loss relative to the PBS control. Interestingly, mice vaccinated with γ-PN alone also showed significant protection relative to the PBS control ($P = 0.0039$). However, γ-PN immunized mice deemed to have succumbed to A/PR8 infection as indicated by their initial body weight loss similarly to that observed with the PBS group (~15% weight loss), but these γ-PN vaccinated mice began to recover by day 8 and regained their body weight by the end of the monitoring period. Thus, these data indicate that co-immunisation with γ-PN + γ-FLU does not impair the ability of the γ-FLU component to provide protection against influenza. Furthermore, the data also show that the γ-PN vaccine may induce non- specific immunity that facilitates recovery from influenza infection.

To further understand the impact of co-immunisation on the nature of anti-FLU responses, the influenza-specific $CD4^+$ T cell memory responses were also investigated. Lung suspensions from co-immunized mice restimulated *ex vivo* with the γ-FLU antigen contained greater frequencies of influenza-specific $CD4^+$ T effector and Th17 cells relative to unvaccinated (PBS) control animals (Figure 5.15A). These populations were also enhanced in

comparison to those from mice vaccinated with γ-FLU alone. The levels of Th17 cells were also significantly increased in spleens of co-immunized mice as were the numbers of $CD8^+$ T effector and Tc1 cells (Figure 5.15B). In addition, the level of Th1 cells in the lungs and spleen from co-immunized mice also appeared to be enhanced relative to other groups, but this did not reach statistical significance. There were also significantly higher concentrations of IL-17 and IFN-γ in supernatants from both the lung and the spleen cell suspensions of the co-immunized group relative to the PBS controls. IL-17 levels were significantly higher relative to γ-FLU vaccine group in spleen cell suspensions, but not the lung (Figure 5.15C). For mice immunized with γ-FLU only, a significant increase in the number of $CD4^+$ T effector cells in the lungs relative to PBS controls was detected. There were no significant increases detected in Th17, Th1, or $CD8^+$ T cell populations (Tc1 or Tc17) in the spleen and lung suspensions. However, the levels of Tc1 from spleen suspensions did appear to be enhanced relative to the PBS control, but this did not reach statistical significance (Figure 5.15A&B). Interestingly, there was a significant elevation in IL-17 and IFN-γ levels in supernatants from the lung (Figure 5.15C). Thus, these data confirm that co-immunisation with γ-PN + γ-FLU enhances the total number of lung influenza-specific $CD4^+$ effector cells and that this is associated with an unexpected increase in the population of Th17 cells.

Lastly, the impact of the combination vaccine on influenza antibody responses was investigated. Sera were collected from immunized or co-immunized mice and analyzed for A/PR8-specific IgG titres by ELISA. Immune sera were also analyzed for neutralising ability using a focus forming inhibition (FFI) assay. The data indicate that co-immunisation does not alter influenza-specific antibody titres as IgG and IgA levels were similar to that observed with individual administration with γ-FLU vaccine (Figure 5.16). In addition, results from the FFI assay revealed that sera from mice vaccinated with either γ-FLU alone or co-immunized with γ-PN showed comparable levels of virus inhibition. The MFI levels (indicating virus infection) for both immunized groups were significantly reduced in comparison to that observed with sera from the PBS-vaccinated group (Figure 5.17). Interestingly, sera from mice vaccinated with γ-PN alone, did not have any neutralising ability as the MFI level was comparable to PBS control mice. These data suggest that co-immunisation with γ-PN + γ-FLU does not affect the neutralising ability of influenza-specific antibodies induced by the γ-FLU vaccine. Taken together, these data further supports the notion that co-immunisation with a combined vaccine to *S. pneumoniae* and influenza does not compromise pathogen-specific immune responses.

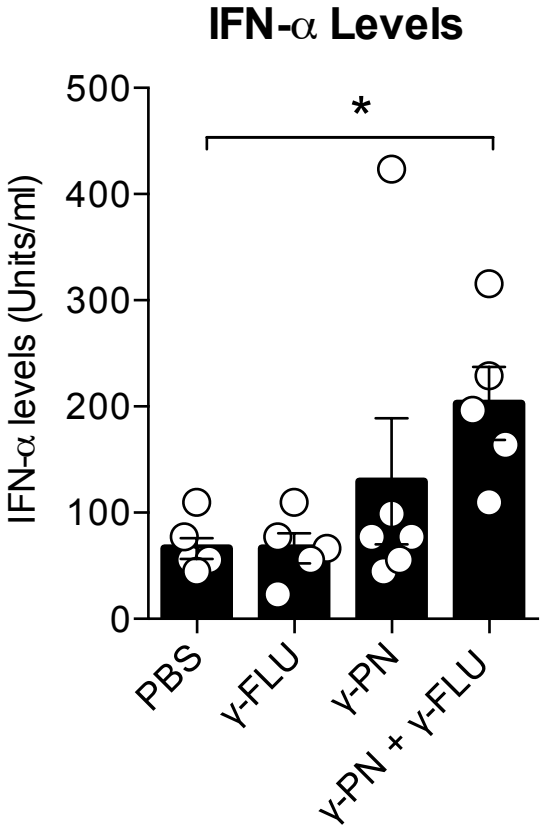


Figure 5.13: Co-vaccination with γ-PN + γ-FLU enhances IFN-I levels following live D39 challenge. Mice (n = 6 per group) were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were challenged IN with D39 (Section 2.14). 48 hours post challenge, sera were harvested from mice and analyzed for the level of IFN-α by ELISA. Results are presented as mean ± SEM. *, $P < 0.05$ (One-way ANOVA).

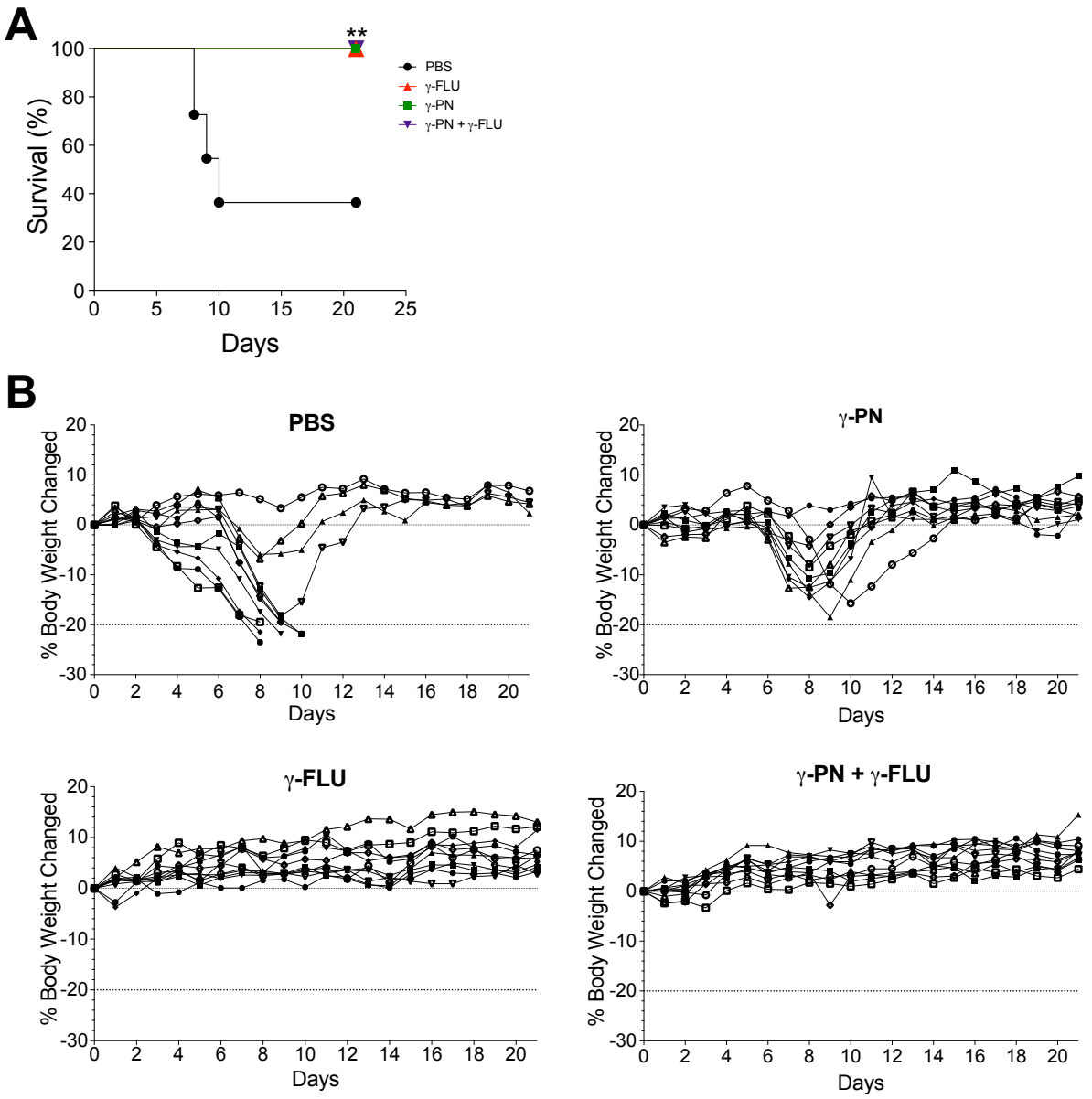
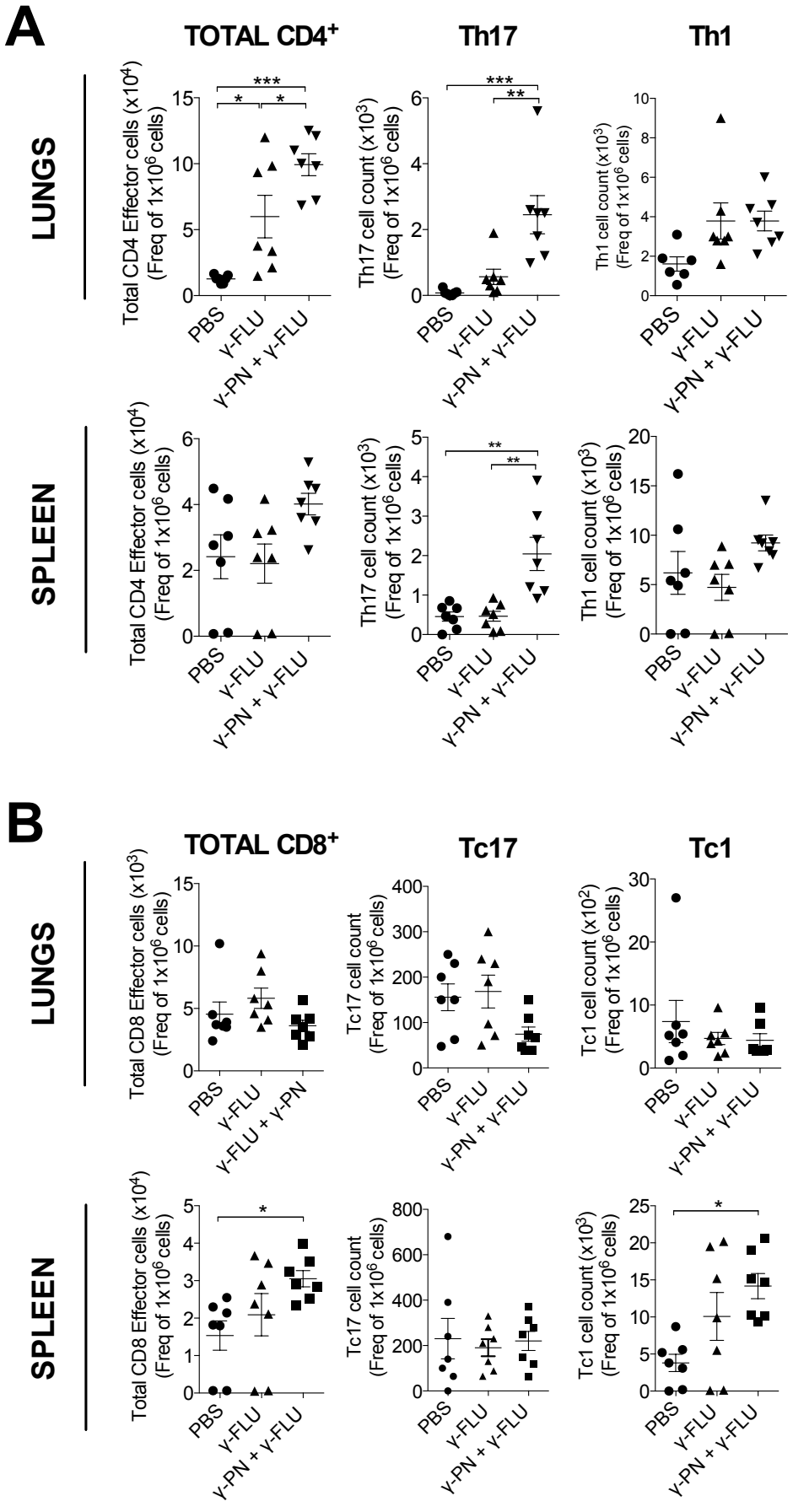


Figure 5.14: Co-immunisation with γ-PN + γ-FLU does not compromise vaccine-induced anti-influenza immunity. Mice (n = 10) were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were challenged IN under anaesthesia with A/PR8 (Section 2.14). (A) Survival rates and (B) percentage weight loss is shown for a period of 21 days. The dotted line represents 20% weight loss (trigger for euthanasia). Data were analyzed using a Fisher exact test (**, $P < 0.01$).


A
TOTAL CD4+
Th17
Th1
LUNGS
SPLEEN
Total CD4 Effector cells (x10^4) (Freq of 1x10^6 cells)
Th17 cell count (x10^3) (Freq of 1x10^6 cells)
Th1 cell count (x10^3) (Freq of 1x10^6 cells)
PBS
γ-FLU
γ-PN + γ-FLU
B
TOTAL CD8+
Tc17
Tc1
Total CD8 Effector cells (x10^3) (Freq of 1x10^6 cells)
Tc17 cell count (Freq of 1x10^6 cells)
Tc1 cell count (x10^2) (Freq of 1x10^6 cells)
γ-FLU + γ-PN
Total CD8 Effector cells (x10^4) (Freq of 1x10^6 cells)
Tc17 cell count (Freq of 1x10^6 cells)
Tc1 cell count (x10^3) (Freq of 1x10^6 cells)

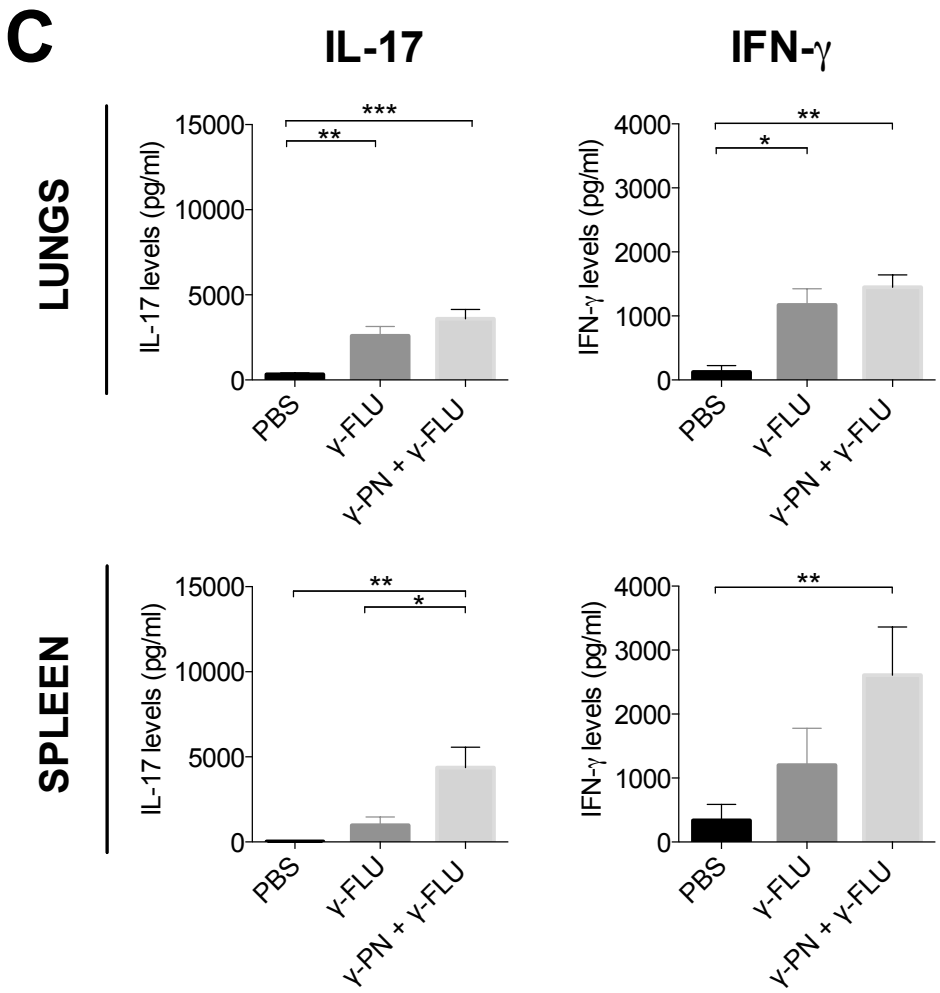


Figure 5.15: Co-immunisation with γ-PN + γ-FLU enhances influenza-specific memory Th17 cells. Mice (n = 7 per group) were immunized IN with 2 doses of γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, the lungs and spleens were harvested and single cell suspensions were restimulated in culture with the γ-FLU vaccine or media alone (negative control) for 72 hours. (A) Frequency of live $CD4^+$ and $CD8^+$ T effector cell subsets in lung and spleen suspensions determined by flow cytometry: Total $CD4^+$ T effector cells ($CD3^+CD4^+CD44^+$), Th17 ($CD3^+CD4^+CD44^+IL\text{-}17^+$) and Th1 ($CD3^+CD4^+CD44^+IFN\text{-}\gamma^+$). (B) Total $CD8^+$ T effector cells ($CD3^+CD8^+CD44^+$), Tc17 ($CD3^+CD8^+CD44^+IL\text{-}17^+$) and Tc1 ($CD3^+CD8^+CD44^+IFN\text{-}\gamma^+$). (C) Levels of cytokines (IL-17, IFN-γ) in the supernatant of cultured lymphocytes from the spleens and the lungs. Data were analyzed using a One-way ANOVA (*, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

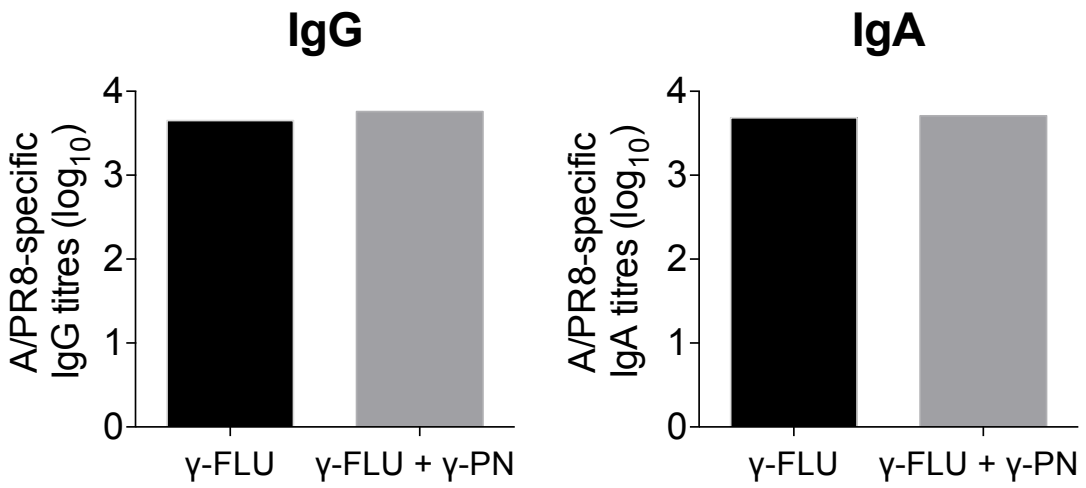
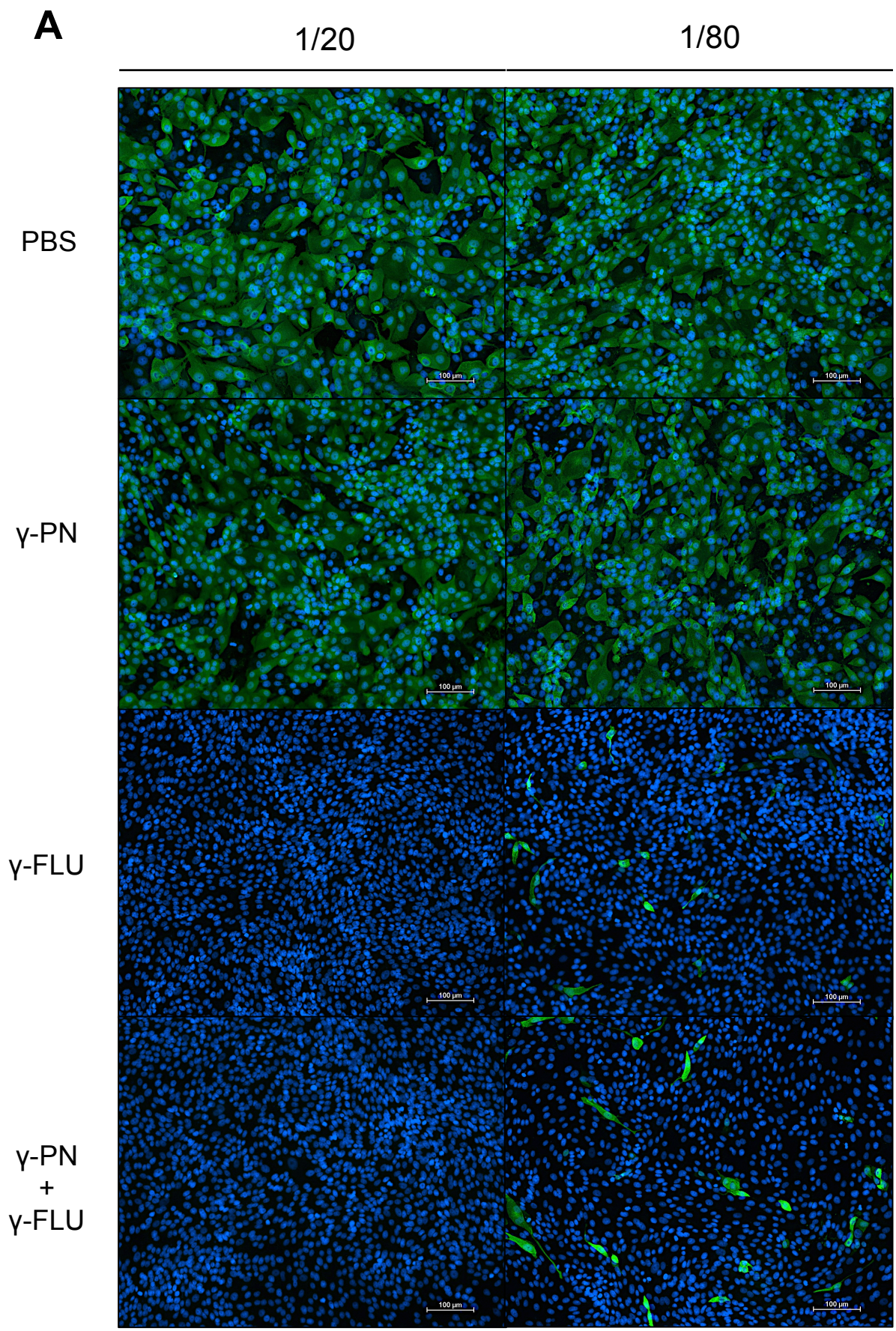


Figure 5.16: Co-immunisation with γ-PN + γ-FLU does not alter the level of influenza-specific humoral responses. Mice (n = 7) were immunized IN with 2 doses of γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, sera were collected and analyzed for influenza-specific IgG and IgA titres by ELISA. Geometric mean for antibody titre is shown.


A
1/20
1/80
PBS
γ-PN
γ-FLU
γ-PN
+
γ-FLU
100 µm

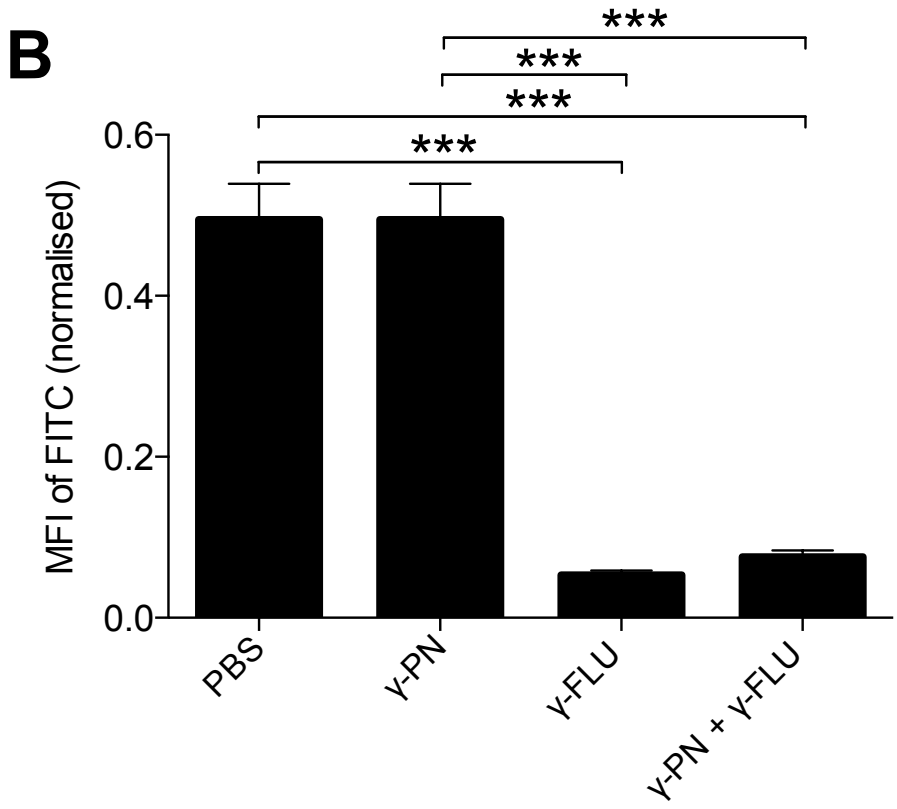


Figure 5.17: Co-vaccination with γ-FLU + γ-PN does not compromise the neutralising ability of influenza-specific antibodies. Mice were immunized IN with 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, sera were collected and analyzed for neutralising ability in a focus forming inhibition assay. (A) Images show MDCK monolayers infected with A/PR8 virus pre-treated with sera at 1/20 and 1/80 dilutions for all groups (Blue = nuclei of MDCK cells, Green = IgG bound to influenza virions) (sera pooled from 10 mice). (B) Graph represents MFI quantification analysis of monolayers infected with virus pre-treated with sera from all groups at 1/80 dilution. Data were analyzed using a One-way ANOVA (*** $P < 0.001$).

5.2.9 Co-immunisation elicits significant protection against co-infection.

It is well known that asymptomatic nasopharyngeal carriage of *S. pneumoniae* can progress into invasive disease following co-infection with influenza. It has previously been shown that co-infection with *S. pneumoniae* with influenza results in enhanced bacterial counts in upper respiratory tract lavages, which is responsible for an exacerbation of infection (412). Therefore, a co-infection model was established to be able to determine the efficacy of the γ-PN/γ-FLU combination vaccine against co-infection. Three groups of mice were colonized intranasally with EF3030 and 4 days later the mice were either anaesthetized and administered a sublethal dose of A/PR8 intranasally (co-infection; PN + FLU) or treated with PBS (to establish pneumonia (PN)). Mice administered PBS intranasally without anaesthetic (NO AN) served as a colonization only control group. 7 days later, CFU recovery was determined in the nasopharynx and lungs. As expected, co-infected mice (PN + FLU) showed greatly enhanced bacterial counts in both the nasopharynx and the lungs relative to mice with PN or the colonization only group (NO AN) (Figure 5.18). This experimental regime confirmed that co-infection with *S. pneumoniae* and influenza exacerbates focal pneumonia.

Using this experimental model of co-infection, the efficacy of the combined γ-PN + γ-FLU vaccine against co-infection was investigated. The data show that mice co-immunized with γ-PN + γ-FLU were the only group that displayed significant protection relative to the unvaccinated (PBS) control mice following co-infection with influenza and *S. pneumoniae* (PN + FLU) (P = 0.0351 for PN + FLU) (Figure 5.19). These data confirm that co-immunisation enhances the vaccine efficacy of γ-PN against pneumococcal pneumonia as well as conferring significant protection against severe co-infection with both *S. pneumoniae* and influenza.

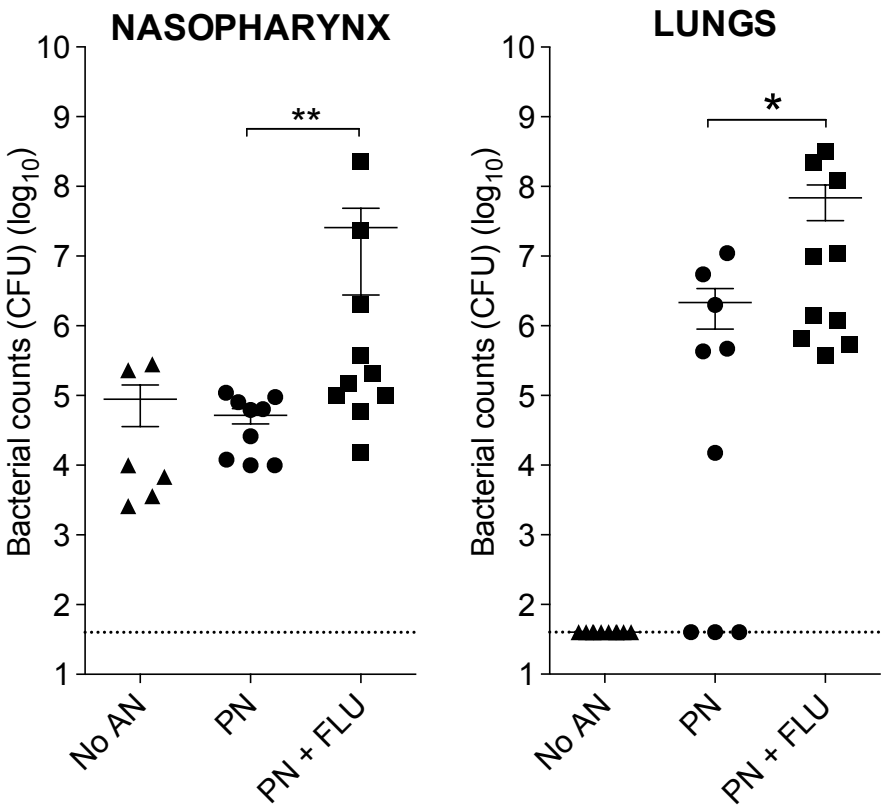


Figure 5.18: Co-infection with influenza and *S. pneumoniae* enhances bacterial loads. Mice (n = ≥ 5) were colonized with EF3030 and 4 days later mice were injected IN with either PBS (PN) or a sublethal dose of A/PR8 (PN + FLU) under anaesthesia (Section 2.14). Mice were also injected IN with PBS without anaesthetic (No AN). 7 days later, the nasopharynx and lungs were harvested to determine bacterial counts. Results are presented as mean ± SEM and analyzed using a One-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

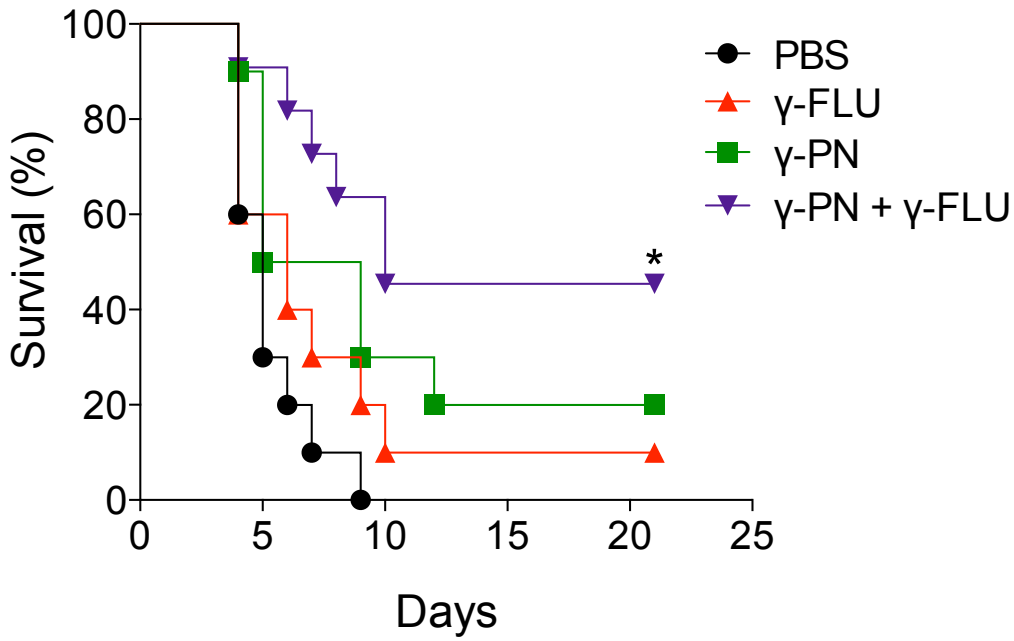


Figure 5.19: Co-immunisation with γ-PN + γ-FLU provides significant protection against co-infection. Mice (n = 10) were immunized IN with either 2 doses of γ-PN, γ-FLU or co-immunized with γ-PN + γ-FLU (Section 2.12). 2 weeks after the second immunisation, mice were infected IN with EF3030 and then 4 days later were given A/PR8 IN (co-infection model) (Section 2.14). Mice were monitored for 21 days and survival rates are shown. Data were analyzed using a Fisher exact test (*, $P < 0.05$).

5.3 Discussion

Current strategies to reduce the risk of co-infection with influenza and *S. pneumoniae* are focused on developing vaccines against individual pathogens. Although patients receiving both vaccinations show reduced viral-associated pneumonia (249, 250, 407), challenges still remain with the current individual vaccines such as serotype-dependence, which allows individual bacteria or viral strains to escape vaccine-induced immunity and cause significant disease.

This study demonstrates that intranasal co-vaccination with γ-PN + γ-FLU does not compromise the protective efficacy of either the γ-PN or γ-FLU vaccine component against intranasal challenge with the pneumococcal strain D39 or the influenza strain A/PR8, respectively. Surprisingly, the data show that combination of the two vaccines significantly enhanced the protective efficacy of the γ-PN vaccine against pneumococcal pneumonia, as well as severe co-infection with *S. pneumoniae* and influenza. To my knowledge, these findings represent the first evidence regarding a mucosal combination vaccine containing whole pneumococcal cells and whole influenza virions that can protect against single and dual infection. Furthermore, the data illustrate the adjuvant-like properties of γ-FLU.

The pneumonia and co-infection models used in these studies rely on the ability of pneumococci to substantially colonize the nasopharynx and migrate into the lungs to establish focal pneumonia upon intranasal delivery of PBS or A/PR8 (in the case of the co-infection model). This experimental design represents a clinically relevant model of bacterial colonisation followed by aspiration, which leads to the establishment of pneumonia. This is different to what is previously shown in Chapter 3 with EF3030. Significant reductions in nasopharyngeal bacterial counts detected in co-immunized mice confirm that the combination vaccine mediates protection at the stage of colonization as well as protecting invasive disease. As mentioned in Section 3.3, eliminating colonization is a double-edged sword as it conveys herd immunity, but vacates a niche that might then be occupied by other pathogens. However, a vaccine that has the ability to significantly reduce bacterial numbers, but not eliminate pneumococcal colonization entirely, may result in significant net benefit in terms of reduced transmission in the community as well as protection from invasive disease with minimal risk of replacement disease.

It has been previously reported that protection against nasopharyngeal carriage is mainly dependent on the induction of Th17 cells with a limited role for B cell responses (84, 85, 367). Previous studies have also reported the efficacy of a CT adjuvanted pneumococcal vaccine to induce Th17-dependent protection against colonization (85, 133). As demonstrated earlier (Chapter 3), the serotype-independent protection elicited by the un-adjuvanted γ-PN vaccine was mediated by both B cells and innate derived IL-17 responses, not Th17 cells. This study demonstrated that co-immunisation resulted in elevated pneumococcus-specific antibody levels, indicating that augmented B cell responses may play a role in the enhanced efficacy of the combined vaccine. This observation also supports previous work illustrating the γ-FLU-mediated enhancement of humoral responses to a co-administered antigen (348). In addition, the data confirm that $CD4^+$ T cells play a major role in the observed enhancement of vaccine efficacy, as humoral immune responses alone were not sufficient to mediate protection in $CD4^+$ depleted mice. In particular, a significant induction of antigen-specific Th17 cells in co-immunized mice was observed in comparison to individual vaccination with γ-PN. Considering the importance of Th17 responses during colonization, it is likely that Th17 cells are responsible for the superior protection against nasopharyngeal carriage observed in mice receiving the combination vaccine. Nevertheless, the augmented antibody responses may still be playing a minor role. In these studies there also appeared to be detectable Th17 levels in cultured lymphocytes from the lungs of γ-PN vaccinated mice, but this was not evident in the lungs following live D39 challenge, which is consistent to that observed earlier in Chapter 3. This may explain why the unadjuvanted γ-PN vaccine alone, compared to the combination vaccine was unable to provide protection against colonization-induced pneumonia.

In addition to IL-17, IFN-γ is also known to be an important mediator of bacterial defence during pneumococcal infection. High IFN-γ levels have been shown to be associated with increased expression of neutrophil chemoattractant CXCL1 to promote neutrophil influx to facilitate pneumococcal clearance (360). Although NK cells (360) and neutrophils (361) have been reported to be major sources of IFN-γ during pneumococcal infection, IFN-γ derived from Th1 cells has also been implicated in defence against colonization (60). Consistent with this, lung suspensions from co-immunized mice restimulated *ex vivo* with the γ-PN antigen also show a significant increase in pneumococcal-specific Th1 cells. These cells also appeared to be increased following live pneumococcal challenge, but this did not reach

statistical significance. Nevertheless Th1 cells may also contribute to the enhanced vaccine efficacy of the γ-PN component of the combination vaccine.

Furthermore, aside from conventional memory T cells, there has been a lot of supporting evidence showing the beneficial role of TRM cells (413). There have been no reports regarding the development of TRM cells during pneumococcal infection nor has this been anticipated as part of pneumococcal vaccine design. As previously shown, the γ-PN vaccine alone does not promote development of TRM memory responses (Chapter 3), however, can promote development of TRM cells when co-administered with CT (Chapter 4). Similarly, as observed in this study, there were significant levels of $CD4^+$ and $CD8^+$ TRM cells in mice co-immunized with γ-PN + γ-FLU. In particular, a profound increase was observed in the level of $CD4^+$ IL-17^+ and $CD4^+$ IFN-γ^+ TRM cells in the lungs 48 hours following live pneumococcal challenge. Although antigen-specificity cannot be determined in these experiments without the use of tetramers or TCR transgenic systems, the detection of high levels of TRM cells in co-immunized mice following pneumococcal challenge suggests that the TRM cells may be pneumococcal-specific. The prior establishment of TRM at the portal of pathogen entry may provide rapid immune responses to elicit optimal protection without relying solely on the recruitment of effector memory responses from the circulatory pool.

It appears that intranasal administration of the combination vaccine could mimic the natural development of TRM cells in the lungs, in contrast to other routes of administration utilised by current licensed pneumococcal vaccines (414). The importance of TRM within immunisation strategies has been previously demonstrated with influenza. Intranasal administration, in contrast to intraperitoneal administration of a sub-lethal recombinant influenza strain WSN-OVA, was shown to induce TRM cells and significantly protect against heterosubtypic challenge, despite both routes producing optimal tissue effector memory cells (209). Therefore, the induction of TRM cells by the combination vaccine in addition to high levels of antibodies, Th17 and Th1 responses, could facilitate the optimal clearance of pneumococci in comparison to individual vaccination with γ-PN, perhaps through IL-17/ IFN-γ driven recruitment of phagocytic cells or antibody-mediated opsonophagocytosis.

This study clearly demonstrates that γ-FLU behaves like an adjuvant by altering the priming micro-environment to enhance and favour immunity against a co-administered antigen, which

in this case is inactivated *S. pneumoniae*. It has been previously reported that γ-FLU is a strong inducer of IFN-I (330) and enhances the immunogenicity of a co-administered viral vaccine most likely through IFN-I responses (348). In the present study, serum IFN-α levels were significantly enhanced in co-immunized mice after live pneumococcal challenge. IFN-I responses have been reported to be important for pneumococcal defence in reducing cell invasion and transmigration across epithelial cells from within the lung into the bloodstream (415). However, in the context of bacterial co-infection with influenza, high IFN-I levels induced by live influenza have been shown to inhibit both Th17 (370) and γδ T cells (239), which may impede anti-bacterial defence. Despite the enhanced levels of IFN-I shown from co-immunized mice, accelerated Th17 responses were observed in co-immunized mice in comparison to single immunisation with γ-FLU, suggesting that an IFN-I-related inhibitory effect on Th17 responses appears to not occur in the context of this vaccination strategy. The data also suggest that IFN-I responses may be a possible component involved in the enhanced efficacy observed in co-immunized mice. Nevertheless, further experiments are required to confirm the role of IFN-I in the enhanced efficacy of the combined vaccine.

In addition, the molecular mechanism by which γ-FLU governs the induction of Th17 responses when in combination with γ-PN was not evaluated in this study. However, previous studies suggest a possible involvement of TLR4. Injection of mice with an inactivated influenza strain H5N1 has been reported to induce the generation of reactive oxygen species that triggers the production of oxidised phospholipids (OPLs) in the lung airways. OPLs were shown to induce the production of IL-6 through TLR4 signalling in alveolar macrophages (416). In addition, the involvement of TLR4 signalling to promote the generation of IL-17 differentiating cells has been reported *in vitro* (417) and *in vivo* (418, 419). In addition, it has also been shown that there is a lack of expansion of Th17 cells in splenocytes from TLR2 KO mice previously colonized with pneumococci in comparison to WT mice, upon ex vivo stimulation. Thus, a role for TLR2 signalling in Th17 cell generation has also been indicated (84). Therefore, vaccination with γ-PN + γ-FLU could promote signalling through TLR4 to induce IL-6, which creates the priming environment for Th17 differentiation, or could involve signalling through TLR2, but both of these proposals remain to be tested.

Interestingly, this study demonstrated that the combination vaccine also induced high levels of antigen-specific Th17 cells and IL-17 in culture supernatants in response to *ex vivo* stimulation with the γ-FLU vaccine antigen. The role of Th17 immunity during influenza

infection is still controversial and it remains unclear whether it is beneficial (420) or detrimental to the host (419, 421). Importantly, the combination vaccine was shown to not adversely affect the immunogenicity or protective efficacy of γ-FLU following live A/PR8 challenge. Although the possible source of the increase in IL-17 levels in cultures related to single immunisation with γ-FLU was not investigated, the induction of Th17 cells in the co-immunized group appears to enhance the protective efficacy of the combination vaccine against pneumococcal challenge without compromising the protective efficacy against lethal influenza challenge. Thus, IL-17 and Th17 responses are not detrimental in γ-FLU induced protective immunity. Interestingly, mice vaccinated with the γ-PN vaccine alone were protected against lethal influenza challenge, despite the apparent weight loss. This suggests the potential development of a non-specific cross-reactive immunity. However, results from the FFI assay reveal that sera from γ-PN vaccinated mice did not exhibit any influenza neutralizing activity, implicating that antibody responses may not be the source of cross-protective immunity to influenza. Future experiments can further evaluate the immune components underlying this non-specific immunity detected in γ-PN vaccinated mice.

In conclusion, this study provides the first evidence illustrating the use of a whole virion influenza vaccine to alter pathogen-specific immunity and to enhance the protective efficacy of a whole-cell pneumococcal vaccine, when used in combination. It was further demonstrated that the combined vaccine can induce effective protection against each individual pathogen, and can provide protection in a severe co-infection model. Considering that the γ-PN and γ-FLU vaccines are able to induce serotype-independent and heterosubtypic protection, respectively, this vaccination strategy heralds the possibility a simplified “universal” immunization strategy for protecting against diverse serotypes and strains of both *S. pneumoniae* and influenza.

CHAPTER 6

Final discussion

S. pneumoniae and Influenza infections represent a significant global burden to the human population. Due to the catastrophic repercussions that resulted from emergence of the 1918 Spanish influenza pandemic and the ongoing clinical synergism of these two pathogens, vaccinologists are determined to generate optimal vaccines to limit the prevalence of individual infection and to overcome the enhanced pathogenesis associated with co-infection.

The promising results of this study strengthen the credibility of employing gamma-irradiation as an inactivation method for development of vaccines against respiratory bacterial pathogens. In addition, these findings further support the effectiveness of gamma-irradiation as a method for inactivation of influenza A virus. To my knowledge, this is the first study to report that gamma-irradiation completely abrogates the replication ability of *S. pneumoniae* whole-cells whilst still maintaining its immunogenicity. The data show that intranasal vaccination with these inactivated bacterial cells induces effective mucosal and systemic immune responses to provide serotype-independent protection against pneumococcal challenge. This vaccination approach represents a much safer and easier alternative to other inactivation approaches such as β-propiolactone, which is associated with possible carcinogenic effects, requires a more complicated purification protocol and has been noted to solubilize proteins. In addition, gamma-irradiation is also a safe option in comparison to live-attenuated vaccines. Despite the efficacy reported with live-attenuated vaccines, there is always the concern that these vaccine strains could revert to a more virulent form and induce adverse side effects due to low-level replication ability (Section 1.1.6.2). As a result, these safety issues have encouraged researchers to focus on the development of completely inactivated pathogens for intranasal delivery. Thus, employing gamma-irradiation as a method to generate an inactivated pneumococcal whole-cell vaccine offers a desirable vaccination approach as it eliminates problems associated with sterility and possible reversion to virulence, the major problems hindering manufacturing of intranasal vaccines.

In addition, it is also important to emphasize that many inactivated vaccine antigens intended for intranasal administration are poorly immunogenic and therefore require the use of adjuvants. Importantly, the efficacy reported previously with intranasal pneumococcal whole-cell vaccines have been illustrated with the administration of CT, which is not suitable for human use. This study demonstrated the ability of non-adjuvanted γ-PN to mediate protection and also demonstrated that co-administration of γ-PN with CT as an adjuvant resulted in

enhanced vaccine efficacy. Strikingly, the data illustrate that γ-PN vaccine immunogenicity and protective efficacy is also significantly enhanced when co-administered with γ-FLU. Thus, vaccination with γ-PN either alone without an adjuvant or co-administered with γ-FLU eliminates any concerns regarding adjuvant-related side effects. This further emphasizes the immunogenic potential of the vaccine and highlights the advantage of this inactivation strategy for the development of a mucosal vaccine against *S. pneumoniae*. Intranasal delivery of vaccines is also desirable as it is relatively quick, painless and easy to administer which is an additional bonus in comparison to receiving multiple injections through parenteral routes. As a result, these experimental findings hold promise for utilizing this inactivation method for other respiratory bacterial pathogens.

As mentioned earlier (Section 1.3), lethal synergism between influenza and *S. pneumoniae* is often associated with significant morbidity and mortality rates worldwide, particularly during the emergence of pandemics. This study presents a novel combination vaccine that can protect against disease caused by both respiratory pathogens and the possibility of co-infection. Various problems associated with current licenced pneumococcal and influenza vaccines limit the ability of these individual vaccines to protect against the prevalence of co-infection, as current individual vaccines are sub-optimal in protecting against all different types of *S. pneumoniae* and influenza. In addition, influenza vaccines need to be reformulated annually which also hinders the ability to generate a combination vaccine against both of these pathogens. Considering that γ-FLU has been reported to be cross protective and that the effectiveness γ-PN has been illustrated in this study, adopting the novel combination vaccination strategy that incorporates both of these vaccines could significantly reduce hospitalizations and mortality associated with co-infection without the need for annual re-formulation. This would have considerable impact on human health, particularly within developing countries where there are significant morbidity and mortality rates for both of these respiratory pathogens. In addition, implementation of this combination vaccine would be advantageous in simplifying childhood immunisation regimes.

This study, to my knowledge is also the first report showing the effectiveness of combining whole inactivated bacterial cells and whole inactivated virion vaccines to protect against individual and co-infection without compromising pathogen-specific immune responses. This is a novel area of investigation in regard to the acceptance of combining intracellular and

extracellular pathogens within a single vaccine formulation. Aside from combination vaccines that contain single antigens intended to primarily induce humoral responses, currently licenced vaccines that incorporate multiple whole microbes are comparable in nature and intended to induce similar immune responses (i.e. immune profiles). For example, the MMR vaccine consists of 3 whole intracellular pathogens that have been reported to induce effective cellular immune responses, which are particularly desirable to defend against these intracellular pathogens (Section 1.4). Given the differences in the specific immune mechanisms that dominate between intracellular and extracellular microbes, it is often assumed that combination of 'dissimilar'' pathogens could antagonize or hinder the development of appropriate immune responses against individual pathogens. However, this study demonstrated that the combination of γ-PN and γ-FLU not only maintains pathogen-specific immune responses, but the γ-FLU vaccine enhances the immunogenicity of the γ-PN vaccine to favour the induction of Th17 and $CD4^+$ TRM cells, which represent the most optimal responses for protection against *S. pneumoniae.* The induction of Th17 cells has previously been reported to be desirable in pneumococcal vaccine design (Section 1.1.5.2). However, the phenomenon of $CD4^+$ TRM cells within pneumococcal infection is novel and has additionally not been reported with pneumococcal vaccination. Although the mechanisms underlying the γ-FLU mediated enhancement of these cellular responses have not been fully investigated due to time constraints, the concept of the adjuvant activity provided by γ-FLU is advantageous and could be exploited within vaccine design to mediate effective immunity against other extracellular pathogens. For example, the enhanced Th17 responses associated with co-immunisation that involve γ-FLU could be evaluated using alternative bacterial respiratory pathogens that are also reliant on Th17 cells for protection such as *S. aureus* (422). In addition, in regard to the adjuvant activity, the major advantage of using γ-FLU is to induce immunity against influenza, which alone is a major global health challenge.

6.1 Future directions.

The most interesting area warranting further investigation in this project, is evaluating the molecular mechanisms of how γ-FLU mediates enhancement of pneumococcal cellular immune responses (TRM and Th17 cells) when co-administered with γ-PN. The observation regarding the enhanced serum IFN-I levels in co-immunized mice only following live pneumococcal challenge suggests a possible involvement of IFN-I. Therefore, future studies should evaluate the importance of IFN-I in mediating enhanced efficacy through use of IFN-I

receptor KO mice or neutralising antibodies to IFN-I. In addition, possible signalling mechanisms involving IFN-I induction such as TLR activation could also be explored as TLRs may be additionally activated or enhanced in the presence of γ-FLU. Such signalling mechanisms could lead to the induction of particular cytokines that polarize the differentiation/maintenance of Th17 and $CD4^+$ TRM memory cell subsets during co-immunisation. It would also be interesting to determine whether the adjuvant properties of γ-FLU requires co-administration with γ-PN, or whether its efficacy is preserved if administered in series with γ-PN. As the concept of combining viral and bacterial inactivated whole pathogens is novel in the field of vaccinology, it would be important to uncover these mechanisms, which could then be potentially exploited for development of combination vaccines with alternative pathogens.

In addition, the strong induction of $CD4^+$ TRM cell responses observed following live pneumococcal challenge also warrants further investigation. Although it was assumed, in this study, that the induced TRM cells observed upon live pneumococcal challenge in co-immunized mice are likely to be pneumococcal-specific, it is necessary to fully confirm antigen-specificity of these TRM cells through the use of techniques such as staining with tetramers or TCR transgenic systems. In addition, it would be important to confirm whether these distinct memory cells naturally develop in response to live infection or whether development of these cells is specifically restricted to γ-FLU-mediated enhanced immunity against the γ-PN vaccine.

In regard to investigating the efficacy of co-immunising mice with γ-PN and γ-FLU, experiments were mainly focused on evaluating the role of γ-FLU as an adjuvant on γ-PN-induced immunity. Although this study analyzed the impact of the combination vaccine on influenza-specific antibody responses and protection against influenza homotypic challenge (A/PR8), future experiments should also evaluate the efficacy of the combination vaccine against influenza heterosubtypic challenges (multiple subtypes). It would also be important to evaluate the type of T cell memory responses (T helper cell subsets and TRM cells) induced in co-immunized mice after live influenza challenge to determine whether there are any alterations relative to individual immunisation with γ-FLU. Considering the scientific aim of developing a single vaccine against both influenza and *S. pneumoniae* pathogens, performing these experiments would be important to ensure that the combination vaccine is able to

provide protection against diverse strains of influenza as well as *S. pneumoniae,* without interfering with pathogen-specific immunity.

Interestingly, the results of this study also found that mice vaccinated with γ-PN were protected following lethal influenza challenge, suggesting that there may be a component within the γ-PN vaccine that provides some cross-protection against influenza. Results from the influenza FFI assay demonstrate that sera from γ-PN vaccinated mice did not show any virus neutralization, suggesting an alternative immune mechanism other than antibody responses could be responsible for the non-specific immunity. Uncovering the molecular mechanisms that govern this non-specific immunity induced by γ-PN would be relatively novel and could possibly be exploited in vaccine regimes in the future.

It is pivotal for a vaccine to induce long lasting immunity without the need for many doses of inoculation. Many researchers have expressed their concerns regarding the need for multiple doses of high concentrated antigens for intranasal delivery. There are challenges associated with intranasal delivery that could interfere with proper absorption of the vaccine into the nasal mucosa. For example, the poor permeability of the nasal cavity lined with hairs and mucus represent physical barriers. In addition, nasal enzymes and the local pH can also have a harmful impact on vaccine absorption (219). Furthermore, a common problem with influenza vaccines is the poor immunogenicity in the elderly (423). Recent reports have indeed outlined the efficacy of an adjuvanted Herpes Zoster subunit vaccine in adults over the age of 50. Efficacy was preserved in patients over 70 years (424). Therefore, evaluating the stability of systemic and mucosal immunity induced by γ-FLU and γ-PN intranasal vaccination over a long duration of time (3—6 months) would be important to ensure that these vaccines are sufficient at promoting long-lived immunity. In addition, it would also be interesting to test vaccine efficacy when administered through parental routes, to allow for comparison with the pneumococcal WCV currently in clinical testing.

Overall, the results of this study demonstrate an important concept related to the applicability of gamma-irradiation as an inactivation method for *S. pneumoniae* to develop a mucosal vaccine. The results also demonstrate that γ-FLU can elicit adjuvant like properties to enhance γ-PN vaccine efficacy, thereby presenting the first report of a combined mucosal vaccine against two notoriously challenging pathogens.

REFERENCES

1. Kadioglu, A., J. N. Weiser, J. C. Paton, and P. W. Andrew. 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nature reviews. Microbiology* 6: 288-301.
2. Obaro, S. K., and S. A. Madhi. 2006. Bacterial pneumonia vaccines and childhood pneumonia: are we winning, refining, or redefining? *Lancet Infect Dis* 6: 150-161.
3. Skov Sorensen, U. B., J. Blom, A. Birch-Andersen, and J. Henrichsen. 1988. Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins, and F antigen in pneumococci. *Infect Immun* 56: 1890-1896.
4. Henrichsen, J. 1995. Six newly recognized types of Streptococcus pneumoniae. *J Clin Microbiol* 33: 2759-2762.
5. Austrian, R. 1981. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. *Rev Infect Dis* 3 Suppl: S1-17.
6. Control, C. f. D., and Prevention. 2011. Epidemiology and prevention of vaccine-preventable diseases. *Washington, DC: Public Health Foundation*.
7. Lipsky, B. A., E. J. Boyko, T. S. Inui, and T. D. Koepsell. 1986. Risk factors for acquiring pneumococcal infections. *Arch Intern Med* 146: 2179-2185.
8. Burman, L. A., R. Norrby, and B. Trollfors. 1985. Invasive pneumococcal infections: incidence, predisposing factors, and prognosis. *Rev Infect Dis* 7: 133-142.
9. Feikin, D. R., C. Feldman, A. Schuchat, and E. N. Janoff. 2004. Global strategies to prevent bacterial pneumonia in adults with HIV disease. *Lancet Infect Dis* 4: 445-455.
10. Cohen, A. L., L. H. Harrison, M. M. Farley, A. L. Reingold, J. Hadler, W. Schaffner, R. Lynfield, A. R. Thomas, M. Campsmith, J. Li, A. Schuchat, M. R. Moore, and T. Active Bacterial Core Surveillance. 2010. Prevention of invasive pneumococcal disease among HIV-infected adults in the era of childhood pneumococcal immunization. *Aids* 24: 2253-2262.
11. Klugman, K. P., S. A. Madhi, and C. Feldman. 2007. HIV and pneumococcal disease. *Current opinion in infectious diseases* 20: 11-15.
12. O'Brien, K. L., M. I. Walters, J. Sellman, P. Quinlisk, H. Regnery, B. Schwartz, and S. F. Dowell. 2000. Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection. *Clin Infect Dis* 30: 784-789.
13. Organization, W. H. 2007. Pneumococcal conjugate vaccine for childhood immunization–WHO position paper. *Wkly Epidemiol Rec* 82: 93-104.
14. O'Brien, K. L., L. J. Wolfson, J. P. Watt, E. Henkle, M. Deloria-Knoll, N. McCall, E. Lee, K. Mulholland, O. S. Levine, T. Cherian, Hib, and T. Pneumococcal Global Burden of Disease Study. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* 374: 893-902.
15. van der Poll, T., and S. M. Opal. 2009. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* 374: 1543-1556.
16. Fedson, D. S., and J. A. Scott. 1999. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. *Vaccine* 17 Suppl 1: S11-18.
17. Vives, M., M. E. Garcia, P. Saenz, M. A. Mora, L. Mata, H. Sabharwal, and C. Svanborg. 1997. Nasopharyngeal colonization in Costa Rican children during the first year of life. *Pediatr Infect Dis J* 16: 852-858.
18. Coles, C. L., R. Kanungo, L. Rahmathullah, R. D. Thulasiraj, J. Katz, M. Santosham, and J. M. Tielsch. 2001. Pneumococcal nasopharyngeal colonization in young South Indian infants. *Pediatr Infect Dis J* 20: 289-295.
19. Gray, B. M., G. M. Converse, 3rd, and H. C. Dillon, Jr. 1980. Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 142: 923-933.

20. Hausdorff, W. P., G. Yothers, R. Dagan, T. Kilpi, S. I. Pelton, R. Cohen, M. R. Jacobs, S. L. Kaplan, C. Levy, E. L. Lopez, E. O. Mason, Jr., V. Syriopoulou, B. Wynne, and J. Bryant. 2002. Multinational study of pneumococcal serotypes causing acute otitis media in children. *Pediatr Infect Dis J* 21: 1008-1016.
21. Weinberger, D. M., K. Trzcinski, Y. J. Lu, D. Bogaert, A. Brandes, J. Galagan, P. W. Anderson, R. Malley, and M. Lipsitch. 2009. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. *PLoS pathogens* 5: e1000476.
22. Randle, E., N. Ninis, and D. Inwald. 2011. Invasive pneumococcal disease. *Archives of disease in childhood. Education and practice edition* 96: 183-190.
23. Mac, L. C., and M. R. Kraus. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. *J Exp Med* 92: 1-9.
24. Nuorti, P. J., J. C. Butler, and R. F. Breiman. 1997. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morbidity and Mortality Weekly Report: Recommendations and Reports*: i-24.
25. Martin, M., J. H. Turco, M. E. Zegans, R. R. Facklam, S. Sodha, J. A. Elliott, J. H. Pryor, B. Beall, D. D. Erdman, Y. Y. Baumgartner, P. A. Sanchez, J. D. Schwartzman, J. Montero, A. Schuchat, and C. G. Whitney. 2003. An outbreak of conjunctivitis due to atypical Streptococcus pneumoniae. *The New England journal of medicine* 348: 1112-1121.
26. Crum, N. F., C. P. Barrozo, F. A. Chapman, M. A. Ryan, and K. L. Russell. 2004. An outbreak of conjunctivitis due to a novel unencapsulated Streptococcus pneumoniae among military trainees. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 39: 1148-1154.
27. Weinberger, D. M., Z. B. Harboe, E. A. Sanders, M. Ndiritu, K. P. Klugman, S. Ruckinger, R. Dagan, R. Adegbola, F. Cutts, H. L. Johnson, K. L. O'Brien, J. A. Scott, and M. Lipsitch. 2010. Association of serotype with risk of death due to pneumococcal pneumonia: a meta-analysis. *Clin Infect Dis* 51: 692-699.
28. Heffron, R. 1939. *Pneumonia: with special reference to pneumococcus lobar pneumonia*. Commonwealth fund.
29. Breiman, R. F., J. S. Spika, V. J. Navarro, P. M. Darden, and C. P. Darby. 1990. Pneumococcal bacteremia in Charleston County, South Carolina. A decade later. *Arch Intern Med* 150: 1401-1405.
30. Hansman, D., and M. Bullen. 1967. A resistant pneumococcus. *The Lancet* 290: 264-265.
31. Appelbaum, P. C. 1992. Antimicrobial resistance in Streptococcus pneumoniae: an overview. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 15: 77-83.
32. Jacobs, M. R., H. J. Koornhof, R. M. Robins-Browne, C. M. Stevenson, Z. A. Vermaak, I. Freiman, G. B. Miller, M. A. Witcomb, M. Isaacson, J. I. Ward, and R. Austrian. 1978. Emergence of multiply resistant pneumococci. *The New England journal of medicine* 299: 735-740.
33. Control, C. f. D. 1977. Multiple-antibiotic resistance of pneumococci-South Africa. *Morbid. Mortal. Weekly Rep* 26: 285-286.
34. Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist, and H. R. Masure. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. *Mol Microbiol* 25: 819-829.
35. Holmes, A. R., R. McNab, K. W. Millsap, M. Rohde, S. Hammerschmidt, J. L. Mawdsley, and H. F. Jenkinson. 2001. The pavA gene of Streptococcus pneumoniae

encodes a fibronectin-binding protein that is essential for virulence. *Molecular microbiology* 41: 1395-1408.
36. Bergmann, S., M. Rohde, G. S. Chhatwal, and S. Hammerschmidt. 2001. alpha-Enolase of Streptococcus pneumoniae is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular microbiology* 40: 1273-1287.
37. Jedrzejas, M. J., L. V. Mello, B. L. de Groot, and S. Li. 2002. Mechanism of hyaluronan degradation by Streptococcus pneumoniae hyaluronate lyase. Structures of complexes with the substrate. *The Journal of biological chemistry* 277: 28287-28297.
38. Tong, H. H., L. E. Blue, M. A. James, and T. F. DeMaria. 2000. Evaluation of the virulence of a Streptococcus pneumoniae neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infect Immun* 68: 921-924.
39. Hammerschmidt, S., S. R. Talay, P. Brandtzaeg, and G. S. Chhatwal. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Mol Microbiol* 25: 1113-1124.
40. Hyams, C., E. Camberlein, J. M. Cohen, K. Bax, and J. S. Brown. 2010. The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun* 78: 704-715.
41. Wartha, F., K. Beiter, B. Albiger, J. Fernebro, A. Zychlinsky, S. Normark, and B. Henriques-Normark. 2007. Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. *Cell Microbiol* 9: 1162-1171.
42. Canvin, J. R., A. P. Marvin, M. Sivakumaran, J. C. Paton, G. J. Boulnois, P. W. Andrew, and T. J. Mitchell. 1995. The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. *J Infect Dis* 172: 119-123.
43. Mitchell, A. M., and T. J. Mitchell. 2010. Streptococcus pneumoniae: virulence factors and variation. *Clin Microbiol Infect* 16: 411-418.
44. Steinfort, C., R. Wilson, T. Mitchell, C. Feldman, A. Rutman, H. Todd, D. Sykes, J. Walker, K. Saunders, P. W. Andrew, and et al. 1989. Effect of Streptococcus pneumoniae on human respiratory epithelium in vitro. *Infect Immun* 57: 2006-2013.
45. Paton, J. C., B. Rowan-Kelly, and A. Ferrante. 1984. Activation of human complement by the pneumococcal toxin pneumolysin. *Infect Immun* 43: 1085-1087.
46. Paton, J. C., and A. Ferrante. 1983. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infect Immun* 41: 1212-1216.
47. Ferrante, A., B. Rowan-Kelly, and J. C. Paton. 1984. Inhibition of in vitro human lymphocyte response by the pneumococcal toxin pneumolysin. *Infect Immun* 46: 585-589.
48. Dessing, M. C., R. A. Hirst, A. F. de Vos, and T. van der Poll. 2009. Role of Toll-like receptors 2 and 4 in pulmonary inflammation and injury induced by pneumolysin in mice. *PLoS One* 4: e7993.
49. Rijneveld, A. W., G. P. van den Dobbelsteen, S. Florquin, T. J. Standiford, P. Speelman, L. van Alphen, and T. van der Poll. 2002. Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice. *The Journal of infectious diseases* 185: 123-126.
50. Ren, B., A. J. Szalai, O. Thomas, S. K. Hollingshead, and D. E. Briles. 2003. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer

virulence to a capsular serotype 3 strain of Streptococcus pneumoniae. *Infection and immunity* 71: 75-85.
51. Hakansson, A., H. Roche, S. Mirza, L. S. McDaniel, A. Brooks-Walter, and D. E. Briles. 2001. Characterization of binding of human lactoferrin to pneumococcal surface protein A. *Infection and immunity* 69: 3372-3381.
52. Shaper, M., S. K. Hollingshead, W. H. Benjamin, Jr., and D. E. Briles. 2004. PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. *Infection and immunity* 72: 5031-5040.
53. Lu, L., Z. Ma, T. S. Jokiranta, A. R. Whitney, F. R. DeLeo, and J. R. Zhang. 2008. Species-specific interaction of Streptococcus pneumoniae with human complement factor H. *Journal of immunology* 181: 7138-7146.
54. Dieudonne-Vatran, A., S. Krentz, A. M. Blom, S. Meri, B. Henriques-Normark, K. Riesbeck, and B. Albiger. 2009. Clinical isolates of Streptococcus pneumoniae bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. *Journal of immunology* 182: 7865-7877.
55. Mitchell, T. J. 2003. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol* 1: 219-230.
56. Koppe, U., N. Suttorp, and B. Opitz. 2012. Recognition of Streptococcus pneumoniae by the innate immune system. *Cell Microbiol* 14: 460-466.
57. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* 163: 1-5.
58. Albiger, B., S. Dahlberg, A. Sandgren, F. Wartha, K. Beiter, H. Katsuragi, S. Akira, S. Normark, and B. Henriques-Normark. 2007. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cell Microbiol* 9: 633-644.
59. Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proceedings of the National Academy of Sciences of the United States of America* 100: 1966-1971.
60. van Rossum, A. M., E. S. Lysenko, and J. N. Weiser. 2005. Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model. *Infection and immunity* 73: 7718-7726.
61. Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli, and R. Landmann. 2002. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *The Journal of infectious diseases* 186: 798-806.
62. Branger, J., S. Knapp, S. Weijer, J. C. Leemans, J. M. Pater, P. Speelman, S. Florquin, and T. van der Poll. 2004. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infection and immunity* 72: 788-794.
63. Dinarello, C. A. 2006. Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. *Am J Clin Nutr* 83: 447S-455S.
64. Sahoo, M., I. Ceballos-Olvera, L. del Barrio, and F. Re. 2011. Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections. *ScientificWorldJournal* 11: 2037-2050.
65. McNeela, E. A., A. Burke, D. R. Neill, C. Baxter, V. E. Fernandes, D. Ferreira, S. Smeaton, R. El-Rachkidy, R. M. McLoughlin, A. Mori, B. Moran, K. A. Fitzgerald, J. Tschopp, V. Petrilli, P. W. Andrew, A. Kadioglu, and E. C. Lavelle. 2010.

Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. *PLoS pathogens* 6: e1001191.
66. Davis, K. M., S. Nakamura, and J. N. Weiser. 2011. Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of S. pneumoniae colonization in mice. *The Journal of clinical investigation* 121: 3666-3676.
67. Goubau, D., J. Rehwinkel, and C. Reis e Sousa. 2010. PYHIN proteins: center stage in DNA sensing. *Nature immunology* 11: 984-986.
68. Fang, R., K. Tsuchiya, I. Kawamura, Y. Shen, H. Hara, S. Sakai, T. Yamamoto, T. Fernandes-Alnemri, R. Yang, E. Hernandez-Cuellar, S. R. Dewamitta, Y. Xu, H. Qu, E. S. Alnemri, and M. Mitsuyama. 2011. Critical roles of ASC inflammasomes in caspase-1 activation and host innate resistance to Streptococcus pneumoniae infection. *Journal of immunology* 187: 4890-4899.
69. Nakasone, C., N. Yamamoto, M. Nakamatsu, T. Kinjo, K. Miyagi, K. Uezu, K. Nakamura, F. Higa, H. Ishikawa, L. O'Brien R, K. Ikuta, M. Kaku, J. Fujita, and K. Kawakami. 2007. Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. *Microbes and infection / Institut Pasteur* 9: 251-258.
70. Farkas, J. 1998. Irradiation as a method for decontaminating food. A review. *Int J Food Microbiol* 44: 189-204.
71. Macleod, C. M., R. G. Hodges, M. Heidelberger, and W. G. Bernhard. 1945. Prevention of Pneumococcal Pneumonia by Immunization with Specific Capsular Polysaccharides. *The Journal of experimental medicine* 82: 445-465.
72. Heidelberger, M., L. C. Mac, and et al. 1946. Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides. *The Journal of experimental medicine* 83: 303-320.
73. Malley, R. 2010. Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development. *J Mol Med (Berl)* 88: 135-142.
74. Rapola, S., V. Jantti, R. Haikala, R. Syrjanen, G. M. Carlone, J. S. Sampson, D. E. Briles, J. C. Paton, A. K. Takala, T. M. Kilpi, and H. Kayhty. 2000. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *The Journal of infectious diseases* 182: 1146-1152.
75. Brooks-Walter, A., D. E. Briles, and S. K. Hollingshead. 1999. The pspC gene of Streptococcus pneumoniae encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infection and immunity* 67: 6533-6542.
76. Talkington, D. F., B. G. Brown, J. A. Tharpe, A. Koenig, and H. Russell. 1996. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). *Microb Pathog* 21: 17-22.
77. Khan, M. N., and M. E. Pichichero. 2013. CD4 T cell memory and antibody responses directed against the pneumococcal histidine triad proteins PhtD and PhtE following nasopharyngeal colonization and immunization and their role in protection against pneumococcal colonization in mice. *Infection and immunity* 81: 3781-3792.
78. Tian, H., S. Weber, P. Thorkildson, T. R. Kozel, and L. A. Pirofski. 2009. Efficacy of opsonic and nonopsonic serotype 3 pneumococcal capsular polysaccharide-specific monoclonal antibodies against intranasal challenge with Streptococcus pneumoniae in mice. *Infection and immunity* 77: 1502-1513.

79. Burns, T., Z. Zhong, M. Steinitz, and L. A. Pirofski. 2003. Modulation of polymorphonuclear cell interleukin-8 secretion by human monoclonal antibodies to type 8 pneumococcal capsular polysaccharide. *Infection and immunity* 71: 6775-6783.
80. Doyle, C. R., and L. A. Pirofski. 2016. Reduction of Streptococcus pneumoniae Colonization and Dissemination by a Nonopsonic Capsular Polysaccharide Antibody. *mBio* 7: e02260-02215.
81. Curtis, M. M., and S. S. Way. 2009. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. *Immunology* 126: 177-185.
82. Malley, R., K. Trzcinski, A. Srivastava, C. M. Thompson, P. W. Anderson, and M. Lipsitch. 2005. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proceedings of the National Academy of Sciences of the United States of America* 102: 4848-4853.
83. Malley, R., A. Srivastava, M. Lipsitch, C. M. Thompson, C. Watkins, A. Tzianabos, and P. W. Anderson. 2006. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. *Infection and immunity* 74: 2187-2195.
84. Zhang, Z., T. B. Clarke, and J. N. Weiser. 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *The Journal of clinical investigation* 119: 1899-1909.
85. Lu, Y. J., J. Gross, D. Bogaert, A. Finn, L. Bagrade, Q. Zhang, J. K. Kolls, A. Srivastava, A. Lundgren, S. Forte, C. M. Thompson, K. F. Harney, P. W. Anderson, M. Lipsitch, and R. Malley. 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS pathogens* 4: e1000159.
86. Moffitt, K. L., T. M. Gierahn, Y. J. Lu, P. Gouveia, M. Alderson, J. B. Flechtner, D. E. Higgins, and R. Malley. 2011. T(H)17-based vaccine design for prevention of Streptococcus pneumoniae colonization. *Cell Host Microbe* 9: 158-165.
87. Moffitt, K., M. Skoberne, A. Howard, L. C. Gavrilescu, T. Gierahn, S. Munzer, B. Dixit, P. Giannasca, J. B. Flechtner, and R. Malley. 2014. Toll-like receptor 2-dependent protection against pneumococcal carriage by immunization with lipidated pneumococcal proteins. *Infection and immunity* 82: 2079-2086.
88. Paton, J. C., P. W. Andrew, G. J. Boulnois, and T. J. Mitchell. 1993. Molecular analysis of the pathogenicity of Streptococcus pneumoniae: the role of pneumococcal proteins. *Annu Rev Microbiol* 47: 89-115.
89. Guckian, J. C., G. D. Christensen, and D. P. Fine. 1980. The role of opsonins in recovery from experimental pneumococcal pneumonia. *J Infect Dis* 142: 175-190.
90. Brown, J. S., T. Hussell, S. M. Gilliland, D. W. Holden, J. C. Paton, M. R. Ehrenstein, M. J. Walport, and M. Botto. 2002. The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 99: 16969-16974.
91. Janoff, E. N., C. Fasching, J. M. Orenstein, J. B. Rubins, N. L. Opstad, and A. P. Dalmasso. 1999. Killing of Streptococcus pneumoniae by capsular polysaccharide-specific polymeric IgA, complement, and phagocytes. *The Journal of clinical investigation* 104: 1139-1147.
92. Szalai, A. J., D. E. Briles, and J. E. Volanakis. 1995. Human C-reactive protein is protective against fatal Streptococcus pneumoniae infection in transgenic mice. *Journal of immunology* 155: 2557-2563.
93. Prellner, K. 1981. C1q binding and complement activation by capsular and cell wall components of S. pneumoniae type XIX. *Acta Pathol Microbiol Scand C* 89: 359-364.

94. Walport, M. J. 2001. Complement. Second of two parts. *The New England journal of medicine* 344: 1140-1144.
95. Robbins, J. B., R. Austrian, C. J. Lee, S. C. Rastogi, G. Schiffman, J. Henrichsen, P. H. Makela, C. V. Broome, R. R. Facklam, R. H. Tiesjema, and 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.
96. Douglas, R. M., J. C. Paton, S. J. Duncan, and D. J. Hansman. 1983. Antibody response to pneumococcal vaccination in children younger than five years of age. *The Journal of infectious diseases* 148: 131-137.
97. Peeters, C. C., A. M. Tenbergen-Meekes, B. Haagmans, D. Evenberg, J. T. Poolman, B. J. Zegers, and G. T. Rijkers. 1991. Pneumococcal conjugate vaccines. *Immunol Lett* 30: 267-274.
98. Weinberger, D. M., R. Malley, and M. Lipsitch. 2011. Serotype replacement in disease after pneumococcal vaccination. *Lancet* 378: 1962-1973.
99. Miller, E., N. J. Andrews, P. A. Waight, M. P. Slack, and R. C. George. 2011. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observational cohort study. *The Lancet. Infectious diseases* 11: 760-768.
100. Veenhoven, R., D. Bogaert, C. Uiterwaal, C. Brouwer, H. Kiezebrink, J. Bruin, I. J. E, P. Hermans, R. de Groot, B. Zegers, W. Kuis, G. Rijkers, A. Schilder, and E. Sanders. 2003. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet* 361: 2189-2195.
101. Bogaert, D., A. van Belkum, M. Sluijter, A. Luijendijk, R. de Groot, H. C. Rumke, H. A. Verbrugh, and P. W. Hermans. 2004. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. *Lancet* 363: 1871-1872.
102. Roche, P. W., V. Krause, H. Cook, J. Barralet, D. Coleman, A. Sweeny, J. Fielding, C. Giele, R. Gilmour, R. Holland, R. Kampen, M. Brown, L. Gilbert, G. Hogg, and D. Murphy. 2008. Invasive pneumococcal disease in Australia, 2006. *Commun Dis Intell Q Rep* 32: 18-30.
103. Williams, S. R., P. J. Mernagh, M. H. Lee, and J. T. Tan. 2011. Changing epidemiology of invasive pneumococcal disease in Australian children after introduction of a 7-valent pneumococcal conjugate vaccine. *Med J Aust* 194: 116-120.
104. Bettinger, J. A., D. W. Scheifele, J. D. Kellner, S. A. Halperin, W. Vaudry, B. Law, and G. Tyrrell. 2010. The effect of routine vaccination on invasive pneumococcal infections in Canadian children, Immunization Monitoring Program, Active 2000-2007. *Vaccine* 28: 2130-2136.
105. Singleton, R. J., T. W. Hennessy, L. R. Bulkow, L. L. Hammitt, T. Zulz, D. A. Hurlburt, J. C. Butler, K. Rudolph, and A. Parkinson. 2007. Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* 297: 1784-1792.
106. Salleras, L., A. Dominguez, P. Ciruela, C. Izquierdo, E. Navas, N. Torner, and E. Borras. 2009. Changes in serotypes causing invasive pneumococcal disease (2005-2007 vs. 1997-1999) in children under 2 years of age in a population with intermediate coverage of the 7-valent pneumococcal conjugated vaccine. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 15: 997-1001.
107. Kaplan, S. L., W. J. Barson, P. L. Lin, S. H. Stovall, J. S. Bradley, T. Q. Tan, J. A. Hoffman, L. B. Givner, and E. O. Mason, Jr. 2010. Serotype 19A Is the most common

serotype causing invasive pneumococcal infections in children. *Pediatrics* 125: 429-436.
108. Hampton, L. M., M. M. Farley, W. Schaffner, A. Thomas, A. Reingold, L. H. Harrison, R. Lynfield, N. M. Bennett, S. Petit, K. Gershman, J. Baumbach, B. Beall, J. Jorgensen, A. Glennen, E. R. Zell, and M. Moore. 2012. Prevention of antibiotic-nonsusceptible Streptococcus pneumoniae with conjugate vaccines. *The Journal of infectious diseases* 205: 401-411.
109. Munoz-Almagro, C., C. Esteva, M. F. de Sevilla, L. Selva, A. Gene, and R. Pallares. 2009. Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. *J Infect* 59: 75-82.
110. Fenoll, A., L. Aguilar, M. J. Gimenez, M. D. Vicioso, O. Robledo, J. J. Granizo, and P. Coronel. 2010. Susceptibility of recently collected Spanish pneumococci nonsusceptible to oral penicillin from serotypes not included in the 7-valent conjugate vaccine. *Antimicrobial agents and chemotherapy* 54: 2696-2698.
111. Dagan, R. 2009. Impact of pneumococcal conjugate vaccine on infections caused by antibiotic-resistant Streptococcus pneumoniae. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 15 Suppl 3: 16-20.
112. Pai, R., M. R. Moore, T. Pilishvili, R. E. Gertz, C. G. Whitney, and B. Beall. 2005. Postvaccine genetic structure of Streptococcus pneumoniae serotype 19A from children in the United States. *The Journal of infectious diseases* 192: 1988-1995.
113. Dagan, R., S. Patterson, C. Juergens, D. Greenberg, N. Givon-Lavi, N. Porat, A. Gurtman, W. C. Gruber, and D. A. Scott. 2013. Comparative immunogenicity and efficacy of 13-valent and 7-valent pneumococcal conjugate vaccines in reducing nasopharyngeal colonization: a randomized double-blind trial. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 57: 952-962.
114. Cohen, R., C. Levy, E. Bingen, M. Koskas, I. Nave, and E. Varon. 2012. Impact of 13-valent pneumococcal conjugate vaccine on pneumococcal nasopharyngeal carriage in children with acute otitis media. *Pediatr Infect Dis J* 31: 297-301.
115. Miller, E., N. J. Andrews, P. A. Waight, M. P. Slack, and R. C. George. 2011. Effectiveness of the new serotypes in the 13-valent pneumococcal conjugate vaccine. *Vaccine* 29: 9127-9131.
116. Moore, M. R., R. Link-Gelles, W. Schaffner, R. Lynfield, C. Lexau, N. M. Bennett, S. Petit, S. M. Zansky, L. H. Harrison, A. Reingold, L. Miller, K. Scherzinger, A. Thomas, M. M. Farley, E. R. Zell, T. H. Taylor, Jr., T. Pondo, L. Rodgers, L. McGee, B. Beall, J. H. Jorgensen, and C. G. Whitney. 2015. Effect of use of 13-valent pneumococcal conjugate vaccine in children on invasive pneumococcal disease in children and adults in the USA: analysis of multisite, population-based surveillance. *The Lancet. Infectious diseases* 15: 301-309.
117. Briles, D. E., E. Ades, J. C. Paton, J. S. Sampson, G. M. Carlone, R. C. Huebner, A. Virolainen, E. Swiatlo, and S. K. Hollingshead. 2000. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of Streptococcus pneumoniae. *Infect Immun* 68: 796-800.
118. Ogunniyi, A. D., R. L. Folland, D. E. Briles, S. K. Hollingshead, and J. C. Paton. 2000. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with Streptococcus pneumoniae. *Infect Immun* 68: 3028-3033.

119. Ogunniyi, A. D., M. Grabowicz, D. E. Briles, J. Cook, and J. C. Paton. 2007. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of Streptococcus pneumoniae. *Infection and immunity* 75: 350-357.
120. Yother, J., and D. E. Briles. 1992. Structural properties and evolutionary relationships of PspA, a surface protein of Streptococcus pneumoniae, as revealed by sequence analysis. *J Bacteriol* 174: 601-609.
121. Mukerji, R., S. Mirza, A. M. Roche, R. W. Widener, C. M. Croney, D. K. Rhee, J. N. Weiser, A. J. Szalai, and D. E. Briles. 2012. Pneumococcal surface protein A inhibits complement deposition on the pneumococcal surface by competing with the binding of C-reactive protein to cell-surface phosphocholine. *Journal of immunology* 189: 5327-5335.
122. Zhang, J. R., K. E. Mostov, M. E. Lamm, M. Nanno, S. Shimida, M. Ohwaki, and E. Tuomanen. 2000. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell* 102: 827-837.
123. Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist, and H. R. Masure. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. *Molecular microbiology* 25: 819-829.
124. Khan, M. N., and M. E. Pichichero. 2012. Vaccine candidates PhtD and PhtE of Streptococcus pneumoniae are adhesins that elicit functional antibodies in humans. *Vaccine* 30: 2900-2907.
125. Plumptre, C. D., A. D. Ogunniyi, and J. C. Paton. 2013. Surface association of Pht proteins of Streptococcus pneumoniae. *Infection and immunity* 81: 3644-3651.
126. Khan, M. N., S. K. Sharma, L. M. Filkins, and M. E. Pichichero. 2012. PcpA of Streptococcus pneumoniae mediates adherence to nasopharyngeal and lung epithelial cells and elicits functional antibodies in humans. *Microbes and infection / Institut Pasteur* 14: 1102-1110.
127. McAllister, L. J., H. J. Tseng, A. D. Ogunniyi, M. P. Jennings, A. G. McEwan, and J. C. Paton. 2004. Molecular analysis of the psa permease complex of Streptococcus pneumoniae. *Molecular microbiology* 53: 889-901.
128. Tseng, H. J., A. G. McEwan, J. C. Paton, and M. P. Jennings. 2002. Virulence of Streptococcus pneumoniae: PsaA mutants are hypersensitive to oxidative stress. *Infection and immunity* 70: 1635-1639.
129. Kim, E. H., S. Y. Choi, M. K. Kwon, T. D. Tran, S. S. Park, K. J. Lee, S. M. Bae, D. E. Briles, and D. K. Rhee. 2012. Streptococcus pneumoniae pep27 mutant as a live vaccine for serotype-independent protection in mice. *Vaccine* 30: 2008-2019.
130. Rosch, J. W., A. R. Iverson, J. Humann, B. Mann, G. Gao, P. Vogel, M. Mina, K. A. Murrah, A. C. Perez, W. Edward Swords, E. I. Tuomanen, and J. A. McCullers. 2014. A live-attenuated pneumococcal vaccine elicits CD4+ T-cell dependent class switching and provides serotype independent protection against acute otitis media. *EMBO Mol Med* 6: 141-154.
131. Ferreira, D. M., D. R. Neill, M. Bangert, J. F. Gritzfeld, N. Green, A. K. Wright, S. H. Pennington, L. Bricio-Moreno, A. T. Moreno, E. N. Miyaji, A. D. Wright, A. M. Collins, D. Goldblatt, A. Kadioglu, and S. B. Gordon. 2013. Controlled human infection and rechallenge with Streptococcus pneumoniae reveals the protective efficacy of carriage in healthy adults. *Am J Respir Crit Care Med* 187: 855-864.
132. Gonzalez, B. E., and M. R. Jacobs. 2013. The potential of human nasal colonization with Streptococcus pneumoniae as a universal pneumococcal vaccine. *Am J Respir Crit Care Med* 187: 794-795.

133. Malley, R., M. Lipsitch, A. Stack, R. Saladino, G. Fleisher, S. Pelton, C. Thompson, D. Briles, and P. Anderson. 2001. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infection and immunity* 69: 4870-4873.
134. Malley, R., S. C. Morse, L. C. Leite, A. P. Areas, P. L. Ho, F. S. Kubrusly, I. C. Almeida, and P. Anderson. 2004. Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. *Infection and immunity* 72: 4290-4292.
135. Xu, X., J. Meng, Y. Wang, J. Zheng, K. Wu, X. Zhang, Y. Yin, and Q. Zhang. 2014. Serotype-independent protection against pneumococcal infections elicited by intranasal immunization with ethanol-killed pneumococcal strain, SPY1. *J Microbiol* 52: 315-323.
136. Lu, Y. J., P. Yadav, J. D. Clements, S. Forte, A. Srivastava, C. M. Thompson, R. Seid, J. Look, M. Alderson, A. Tate, J. F. Maisonneuve, G. Robertson, P. W. Anderson, and R. Malley. 2010. Options for inactivation, adjuvant, and route of topical administration of a killed, unencapsulated pneumococcal whole-cell vaccine. *Clin Vaccine Immunol* 17: 1005-1012.
137. Moffitt, K. L., P. Yadav, D. M. Weinberger, P. W. Anderson, and R. Malley. 2012. Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. *Vaccine* 30: 4316-4322.
138. Goncalves, V. M., W. O. Dias, I. B. Campos, C. Liberman, M. E. Sbrogio-Almeida, E. P. Silva, C. P. Cardoso, Jr., M. Alderson, G. Robertson, J. F. Maisonneuve, A. Tate, P. Anderson, R. Malley, F. Fratelli, and L. C. Leite. 2014. Development of a whole cell pneumococcal vaccine: BPL inactivation, cGMP production, and stability. *Vaccine* 32: 1113-1120.
139. Lu, Y. J., L. Leite, V. M. Goncalves, O. Dias Wde, C. Liberman, F. Fratelli, M. Alderson, A. Tate, J. F. Maisonneuve, G. Robertson, R. Graca, S. Sayeed, C. M. Thompson, P. Anderson, and R. Malley. 2010. GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. *Vaccine* 28: 7468-7475.
140. World Health Organization. 2014. Influenza (seasonal).
141. Sridhar, S., K. A. Brokstad, and R. J. Cox. 2015. Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines (Basel)* 3: 373-389.
142. Taubenberger, J. K., and D. M. Morens. 2008. The pathology of influenza virus infections. *Annu Rev Pathol* 3: 499-522.
143. Simonsen, L., L. Schonberger, D. Stroup, N. Arden, and N. Cox. 1996. The impact of influenza on mortality in the USA. *Options for the control of influenza III. Amsterdam: Elsevier Science BV*: 26-33.
144. Kilbourne, E. D. 2006. Influenza pandemics of the 20th century. *Emerging infectious diseases* 12: 9-14.
145. Taubenberger, J. K., and D. M. Morens. 2006. 1918 Influenza: the mother of all pandemics. *Emerging infectious diseases* 12: 15-22.
146. Trampuz, A., R. M. Prabhu, T. F. Smith, and L. M. Baddour. 2004. Avian influenza: a new pandemic threat? *Mayo Clin Proc* 79: 523-530; quiz 530.
147. Patterson, K. D., and G. F. Pyle. 1991. The geography and mortality of the 1918 influenza pandemic. *Bull Hist Med* 65: 4-21.
148. Johnson, N. P., and J. Mueller. 2002. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull Hist Med* 76: 105-115.

149. Neumann, G., and Y. Kawaoka. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. *Emerg Infect Dis* 12: 881-886.
150. Dangi, T., and A. Jain. 2012. Influenza virus: a brief overview. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* 82: 111-121.
151. Obenauer, J. C., J. Denson, P. K. Mehta, X. Su, S. Mukatira, D. B. Finkelstein, X. Xu, J. Wang, J. Ma, Y. Fan, K. M. Rakestraw, R. G. Webster, E. Hoffmann, S. Krauss, J. Zheng, Z. Zhang, and C. W. Naeve. 2006. Large-scale sequence analysis of avian influenza isolates. *Science* 311: 1576-1580.
152. Nelson, M. I., and E. C. Holmes. 2007. The evolution of epidemic influenza. *Nature reviews. Genetics* 8: 196-205.
153. Van Reeth, K. 2000. Cytokines in the pathogenesis of influenza. *Veterinary microbiology* 74: 109-116.
154. Wood, J. M., and J. S. Robertson. 2004. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. *Nat Rev Microbiol* 2: 842-847.
155. Zambon, M. C. 2001. The pathogenesis of influenza in humans. *Rev Med Virol* 11: 227-241.
156. Kash, J. C., and J. K. Taubenberger. 2015. The role of viral, host, and secondary bacterial factors in influenza pathogenesis. *The American journal of pathology* 185: 1528-1536.
157. Bussey, K. A., T. L. Bousse, E. A. Desmet, B. Kim, and T. Takimoto. 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. *Journal of virology* 84: 4395-4406.
158. Song, M. S., P. N. Pascua, J. H. Lee, Y. H. Baek, O. J. Lee, C. J. Kim, H. Kim, R. J. Webby, R. G. Webster, and Y. K. Choi. 2009. The polymerase acidic protein gene of influenza a virus contributes to pathogenicity in a mouse model. *Journal of virology* 83: 12325-12335.
159. O'Neill, R. E., J. Talon, and P. Palese. 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *The EMBO journal* 17: 288-296.
160. Palese, P. ML 2007. Orthomyxoviridae: the viruses and their replication. *Fields Virology*: 1647-1689.
161. Mibayashi, M., L. Martinez-Sobrido, Y. M. Loo, W. B. Cardenas, M. Gale, Jr., and A. Garcia-Sastre. 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol* 81: 514-524.
162. Zamarin, D., A. Garcia-Sastre, X. Xiao, R. Wang, and P. Palese. 2005. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. *PLoS pathogens* 1: e4.
163. Guillot, L., R. Le Goffic, S. Bloch, N. Escriou, S. Akira, M. Chignard, and M. Si-Tahar. 2005. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *The Journal of biological chemistry* 280: 5571-5580.
164. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529-1531.
165. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314: 997-1001.
166. Coates, B. M., K. L. Staricha, K. M. Wiese, and K. M. Ridge. 2015. Influenza A Virus Infection, Innate Immunity, and Childhood. *JAMA Pediatr* 169: 956-963.

167. Garcia-Sastre, A., and C. A. Biron. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* 312: 879-882.
168. Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34: 637-650.
169. Iwasaki, A., and P. S. Pillai. 2014. Innate immunity to influenza virus infection. *Nature reviews. Immunology* 14: 315-328.
170. Li, S., J. Y. Min, R. M. Krug, and G. C. Sen. 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 349: 13-21.
171. Gack, M. U., R. A. Albrecht, T. Urano, K. S. Inn, I. C. Huang, E. Carnero, M. Farzan, S. Inoue, J. U. Jung, and A. Garcia-Sastre. 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 5: 439-449.
172. Ichinohe, T., H. K. Lee, Y. Ogura, R. Flavell, and A. Iwasaki. 2009. Inflammasome recognition of influenza virus is essential for adaptive immune responses. *The Journal of experimental medicine* 206: 79-87.
173. Ichinohe, T., I. K. Pang, and A. Iwasaki. 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nature immunology* 11: 404-410.
174. McAuley, J. L., M. D. Tate, C. J. MacKenzie-Kludas, A. Pinar, W. Zeng, A. Stutz, E. Latz, L. E. Brown, and A. Mansell. 2013. Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. *PLoS pathogens* 9: e1003392.
175. Thomas, P. G., P. Dash, J. R. Aldridge, Jr., A. H. Ellebedy, C. Reynolds, A. J. Funk, W. J. Martin, M. Lamkanfi, R. J. Webby, K. L. Boyd, P. C. Doherty, and T. D. Kanneganti. 2009. The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30: 566-575.
176. Bergsbaken, T., S. L. Fink, and B. T. Cookson. 2009. Pyroptosis: host cell death and inflammation. *Nature reviews. Microbiology* 7: 99-109.
177. Mozdzanowska, K., J. Feng, M. Eid, G. Kragol, M. Cudic, L. Otvos, Jr., and W. Gerhard. 2003. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. *Vaccine* 21: 2616-2626.
178. Topham, D. J., R. A. Tripp, A. M. Hamilton-Easton, S. R. Sarawar, and P. C. Doherty. 1996. Quantitative analysis of the influenza virus-specific CD4+ T cell memory in the absence of B cells and Ig. *J Immunol* 157: 2947-2952.
179. de Jong, J. C., A. M. Palache, W. E. Beyer, G. F. Rimmelzwaan, A. C. Boon, and A. D. Osterhaus. 2003. Haemagglutination-inhibiting antibody to influenza virus. *Dev Biol (Basel)* 115: 63-73.
180. Tamura, S., T. Iwasaki, A. H. Thompson, H. Asanuma, Z. Chen, Y. Suzuki, C. Aizawa, and T. Kurata. 1998. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. *The Journal of general virology* 79 (Pt 2): 291-299.
181. Schulman, J. L., M. Khakpour, and E. D. Kilbourne. 1968. Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. *Journal of virology* 2: 778-786.
182. Ramos, E. L., J. L. Mitcham, T. D. Koller, A. Bonavia, D. W. Usner, G. Balaratnam, P. Fredlund, and K. M. Swiderek. 2015. Efficacy and safety of treatment with an anti-m2e monoclonal antibody in experimental human influenza. *The Journal of infectious diseases* 211: 1038-1044.

183. Asahi, Y., T. Yoshikawa, I. Watanabe, T. Iwasaki, H. Hasegawa, Y. Sato, S. Shimada, M. Nanno, Y. Matsuoka, M. Ohwaki, Y. Iwakura, Y. Suzuki, C. Aizawa, T. Sata, T. Kurata, and S. Tamura. 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. *Journal of immunology* 168: 2930-2938.
184. Yewdell, J. W., R. G. Webster, and W. U. Gerhard. 1979. Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. *Nature* 279: 246-248.
185. Wilson, I. A., and N. J. Cox. 1990. Structural basis of immune recognition of influenza virus hemagglutinin. *Annu Rev Immunol* 8: 737-771.
186. Tamura, S., H. Funato, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, and T. Kurata. 1991. Cross-protection against influenza A virus infection by passively transferred respiratory tract IgA antibodies to different hemagglutinin molecules. *European journal of immunology* 21: 1337-1344.
187. Ekiert, D. C., A. K. Kashyap, J. Steel, A. Rubrum, G. Bhabha, R. Khayat, J. H. Lee, M. A. Dillon, R. E. O'Neil, A. M. Faynboym, M. Horowitz, L. Horowitz, A. B. Ward, P. Palese, R. Webby, R. A. Lerner, R. R. Bhatt, and I. A. Wilson. 2012. Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* 489: 526-532.
188. Bender, B. S., and P. A. Small, Jr. 1992. Influenza: pathogenesis and host defense. *Semin Respir Infect* 7: 38-45.
189. Hou, S., P. C. Doherty, M. Zijlstra, R. Jaenisch, and J. M. Katz. 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J Immunol* 149: 1319-1325.
190. Johnson, B. J., E. O. Costelloe, D. R. Fitzpatrick, J. B. Haanen, T. N. Schumacher, L. E. Brown, and A. Kelso. 2003. Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-infected mice. *Proceedings of the National Academy of Sciences of the United States of America* 100: 2657-2662.
191. Topham, D. J., R. A. Tripp, and P. C. Doherty. 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *Journal of immunology* 159: 5197-5200.
192. Epstein, S. L., C. Y. Lo, J. A. Misplon, and J. R. Bennink. 1998. Mechanism of protective immunity against influenza virus infection in mice without antibodies. *J Immunol* 160: 322-327.
193. Teijaro, J. R., D. Verhoeven, C. A. Page, D. Turner, and D. L. Farber. 2010. Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. *Journal of virology* 84: 9217-9226.
194. Brown, D. M., A. M. Dilzer, D. L. Meents, and S. L. Swain. 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. *Journal of immunology* 177: 2888-2898.
195. Leon, B., J. E. Bradley, F. E. Lund, T. D. Randall, and A. Ballesteros-Tato. 2014. FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun* 5: 3495.
196. McHeyzer-Williams, M., S. Okitsu, N. Wang, and L. McHeyzer-Williams. 2012. Molecular programming of B cell memory. *Nature reviews. Immunology* 12: 24-34.
197. Cella, M., F. Facchetti, A. Lanzavecchia, and M. Colonna. 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nature immunology* 1: 305-310.

198. Ada, G. L., and P. D. Jones. 1986. The immune response to influenza infection. *Current topics in microbiology and immunology* 128: 1-54.
199. Weiss, I. D., O. Wald, H. Wald, K. Beider, M. Abraham, E. Galun, A. Nagler, and A. Peled. 2010. IFN-gamma treatment at early stages of influenza virus infection protects mice from death in a NK cell-dependent manner. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 30: 439-449.
200. Baumgarth, N., and A. Kelso. 1996. In vivo blockade of gamma interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue. *Journal of virology* 70: 4411-4418.
201. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-478.
202. Purwar, R., J. Campbell, G. Murphy, W. G. Richards, R. A. Clark, and T. S. Kupper. 2011. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PloS one* 6: e16245.
203. Gebhardt, T., L. M. Wakim, L. Eidsmo, P. C. Reading, W. R. Heath, and F. R. Carbone. 2009. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nature immunology* 10: 524-530.
204. Masopust, D., D. Choo, V. Vezys, E. J. Wherry, J. Duraiswamy, R. Akondy, J. Wang, K. A. Casey, D. L. Barber, K. S. Kawamura, K. A. Fraser, R. J. Webby, V. Brinkmann, E. C. Butcher, K. A. Newell, and R. Ahmed. 2010. Dynamic T cell migration program provides resident memory within intestinal epithelium. *The Journal of experimental medicine* 207: 553-564.
205. Wakim, L. M., A. Woodward-Davis, and M. J. Bevan. 2010. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proceedings of the National Academy of Sciences of the United States of America* 107: 17872-17879.
206. Mackay, L. K., A. Rahimpour, J. Z. Ma, N. Collins, A. T. Stock, M. L. Hafon, J. Vega-Ramos, P. Lauzurica, S. N. Mueller, T. Stefanovic, D. C. Tscharke, W. R. Heath, M. Inouye, F. R. Carbone, and T. Gebhardt. 2013. The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nature immunology* 14: 1294-1301.
207. Turner, D. L., K. L. Bickham, J. J. Thome, C. Y. Kim, F. D'Ovidio, E. J. Wherry, and D. L. Farber. 2014. Lung niches for the generation and maintenance of tissue-resident memory T cells. *Mucosal Immunol* 7: 501-510.
208. Laidlaw, B. J., N. Zhang, H. D. Marshall, M. M. Staron, T. Guan, Y. Hu, L. S. Cauley, J. Craft, and S. M. Kaech. 2014. CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. *Immunity* 41: 633-645.
209. Wu, T., Y. Hu, Y. T. Lee, K. R. Bouchard, A. Benechet, K. Khanna, and L. S. Cauley. 2014. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *Journal of leukocyte biology* 95: 215-224.
210. Teijaro, J. R., D. Turner, Q. Pham, E. J. Wherry, L. Lefrancois, and D. L. Farber. 2011. Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *Journal of immunology* 187: 5510-5514.
211. Lipatov, A. S., E. A. Govorkova, R. J. Webby, H. Ozaki, M. Peiris, Y. Guan, L. Poon, and R. G. Webster. 2004. Influenza: emergence and control. *J Virol* 78: 8951-8959.

212. Wiley, D. C., and J. J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem* 56: 365-394.
213. Budowsky, E. I., A. Smirnov Yu, and S. F. Shenderovich. 1993. Principles of selective inactivation of viral genome. VIII. The influence of beta-propiolactone on immunogenic and protective activities of influenza virus. *Vaccine* 11: 343-348.
214. Tamura, S., and T. Kurata. 2004. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn J Infect Dis* 57: 236-247.
215. Couch, R. B., and J. A. Kasel. 1983. Immunity to influenza in man. *Annu Rev Microbiol* 37: 529-549.
216. Murphy, B. R., and M. L. Clements. 1989. The systemic and mucosal immune response of humans to influenza A virus. *Curr Top Microbiol Immunol* 146: 107-116.
217. Dhere, R., L. Yeolekar, P. Kulkarni, R. Menon, V. Vaidya, M. Ganguly, P. Tyagi, P. Barde, and S. Jadhav. 2011. A pandemic influenza vaccine in India: from strain to sale within 12 months. *Vaccine* 29 Suppl 1: A16-21.
218. Grohskopf, L. A., S. J. Olsen, L. Z. Sokolow, J. S. Bresee, N. J. Cox, K. R. Broder, R. A. Karron, and E. B. Walter. 2014. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP) -- United States, 2014-15 influenza season. *MMWR Morb Mortal Wkly Rep* 63: 691-697.
219. Riese, P., P. Sakthivel, S. Trittel, and C. A. Guzman. 2014. Intranasal formulations: promising strategy to deliver vaccines. *Expert opinion on drug delivery* 11: 1619-1634.
220. Coelingh, K. L., C. J. Luke, H. Jin, and K. R. Talaat. 2014. Development of live attenuated influenza vaccines against pandemic influenza strains. *Expert Rev Vaccines* 13: 855-871.
221. Vasu, N., G. Ghaffari, E. T. Craig, and T. J. Craig. 2008. Adverse events associated with intranasal influenza vaccine in the United States. *Ther Adv Respir Dis* 2: 193-198.
222. Alsharifi, M., Y. Furuya, T. R. Bowden, M. Lobigs, A. Koskinen, M. Regner, L. Trinidad, D. B. Boyle, and A. Mullbacher. 2009. Intranasal flu vaccine protective against seasonal and H5N1 avian influenza infections. *PLoS One* 4: e5336.
223. Furuya, Y., J. Chan, M. Regner, M. Lobigs, A. Koskinen, T. Kok, J. Manavis, P. Li, A. Mullbacher, and M. Alsharifi. 2010. Cytotoxic T cells are the predominant players providing cross-protective immunity induced by {gamma}-irradiated influenza A viruses. *J Virol* 84: 4212-4221.
224. Alsharifi, M., and A. Mullbacher. 2010. The gamma-irradiated influenza vaccine and the prospect of producing safe vaccines in general. *Immunol Cell Biol* 88: 103-104.
225. Morens, D. M., J. K. Taubenberger, and A. S. Fauci. 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *The Journal of infectious diseases* 198: 962-970.
226. Chien, Y. W., K. P. Klugman, and D. M. Morens. 2009. Bacterial pathogens and death during the 1918 influenza pandemic. *The New England journal of medicine* 361: 2582-2583.
227. Trotter, Y., Jr., F. L. Dunn, R. H. Drachman, D. A. Henderson, M. Pizzi, and A. D. Langmuir. 1959. Asian influenza in the United States, 1957-1958. *Am J Hyg* 70: 34-50.
228. Dauer, C. C. 1958. Mortality in the 1957-58 influenza epidemic. *Public Health Rep* 73: 803-810.
229. Dawood, F. S., A. D. Iuliano, C. Reed, M. I. Meltzer, D. K. Shay, P. Y. Cheng, D. Bandaranayake, R. F. Breiman, W. A. Brooks, P. Buchy, D. R. Feikin, K. B. Fowler,

A. Gordon, N. T. Hien, P. Horby, Q. S. Huang, M. A. Katz, A. Krishnan, R. Lal, J. M. Montgomery, K. Molbak, R. Pebody, A. M. Presanis, H. Razuri, A. Steens, Y. O. Tinoco, J. Wallinga, H. Yu, S. Vong, J. Bresee, and M. A. Widdowson. 2012. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *The Lancet. Infectious diseases* 12: 687-695.

230. Shieh, W. J., D. M. Blau, A. M. Denison, M. Deleon-Carnes, P. Adem, J. Bhatnagar, J. Sumner, L. Liu, M. Patel, B. Batten, P. Greer, T. Jones, C. Smith, J. Bartlett, J. Montague, E. White, D. Rollin, R. Gao, C. Seales, H. Jost, M. Metcalfe, C. S. Goldsmith, C. Humphrey, A. Schmitz, C. Drew, C. Paddock, T. M. Uyeki, and S. R. Zaki. 2010. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *The American journal of pathology* 177: 166-175.
231. Kash, J. C., K. A. Walters, A. S. Davis, A. Sandouk, L. M. Schwartzman, B. W. Jagger, D. S. Chertow, Q. Li, R. E. Kuestner, A. Ozinsky, and J. K. Taubenberger. 2011. Lethal synergism of 2009 pandemic H1N1 influenza virus and Streptococcus pneumoniae coinfection is associated with loss of murine lung repair responses. *MBio* 2.
232. Plotkowski, M. C., E. Puchelle, G. Beck, J. Jacquot, and C. Hannoun. 1986. Adherence of type I Streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR8 virus. *Am Rev Respir Dis* 134: 1040-1044.
233. McCullers, J. A., and K. C. Bartmess. 2003. Role of neuraminidase in lethal synergism between influenza virus and Streptococcus pneumoniae. *The Journal of infectious diseases* 187: 1000-1009.
234. van der Sluijs, K. F., L. J. van Elden, M. Nijhuis, R. Schuurman, S. Florquin, T. Shimizu, S. Ishii, H. M. Jansen, R. Lutter, and T. van der Poll. 2006. Involvement of the platelet-activating factor receptor in host defense against Streptococcus pneumoniae during postinfluenza pneumonia. *Am J Physiol Lung Cell Mol Physiol* 290: L194-199.
235. Hirst, R. A., A. Kadioglu, C. O'Callaghan, and P. W. Andrew. 2004. The role of pneumolysin in pneumococcal pneumonia and meningitis. *Clin Exp Immunol* 138: 195-201.
236. McAuley, J. L., J. E. Chipuk, K. L. Boyd, N. Van De Velde, D. R. Green, and J. A. McCullers. 2010. PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. *PLoS pathogens* 6: e1001014.
237. McAuley, J. L., F. Hornung, K. L. Boyd, A. M. Smith, R. McKeon, J. Bennink, J. W. Yewdell, and J. A. McCullers. 2007. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. *Cell Host Microbe* 2: 240-249.
238. Shahangian, A., E. K. Chow, X. Tian, J. R. Kang, A. Ghaffari, S. Y. Liu, J. A. Belperio, G. Cheng, and J. C. Deng. 2009. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest* 119: 1910-1920.
239. Li, W., B. Moltedo, and T. M. Moran. 2012. Type I interferon induction during influenza virus infection increases susceptibility to secondary Streptococcus pneumoniae infection by negative regulation of gammadelta T cells. *Journal of virology* 86: 12304-12312.
240. Cao, J., D. Wang, F. Xu, Y. Gong, H. Wang, Z. Song, D. Li, H. Zhang, L. Zhang, Y. Xia, H. Xu, X. Lai, S. Lin, X. Zhang, G. Ren, Y. Dai, and Y. Yin. 2014. Activation of IL-27 signalling promotes development of postinfluenza pneumococcal pneumonia. *EMBO Mol Med* 6: 120-140.

241. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology* 19: 683-765.
242. van der Sluijs, K. F., L. J. van Elden, M. Nijhuis, R. Schuurman, J. M. Pater, S. Florquin, M. Goldman, H. M. Jansen, R. Lutter, and T. van der Poll. 2004. IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *Journal of immunology* 172: 7603-7609.
243. Sun, K., and D. W. Metzger. 2008. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. *Nat Med* 14: 558-564.
244. Didierlaurent, A., J. Goulding, S. Patel, R. Snelgrove, L. Low, M. Bebien, T. Lawrence, L. S. van Rijt, B. N. Lambrecht, J. C. Sirard, and T. Hussell. 2008. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. *The Journal of experimental medicine* 205: 323-329.
245. Ghoneim, H. E., P. G. Thomas, and J. A. McCullers. 2013. Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *Journal of immunology* 191: 1250-1259.
246. Mina, M. J., and K. P. Klugman. 2014. The role of influenza in the severity and transmission of respiratory bacterial disease. *The Lancet. Respiratory medicine* 2: 750-763.
247. Madhi, S. A., and K. P. Klugman. 2004. A role for Streptococcus pneumoniae in virus-associated pneumonia. *Nature medicine* 10: 811-813.
248. Ozgur, S. K., U. Beyazova, Y. K. Kemaloglu, I. Maral, F. Sahin, A. D. Camurdan, Y. Kizil, E. Dinc, and H. Tuzun. 2006. Effectiveness of inactivated influenza vaccine for prevention of otitis media in children. *Pediatr Infect Dis J* 25: 401-404.
249. 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: S91-93.
250. Christenson, B., J. Hedlund, P. Lundbergh, and A. Ortqvist. 2004. Additive preventive effect of influenza and pneumococcal vaccines in elderly persons. *Eur Respir J* 23: 363-368.
251. Dominguez, A., J. Castilla, P. Godoy, M. Delgado-Rodriguez, M. Saez, N. Soldevila, J. Astray, J. M. Mayoral, V. Martin, J. M. Quintana, F. Gonzalez-Candelas, J. C. Galan, S. Tamames, A. Castro, M. Baricot, O. Garin, T. Pumarola, C. Cases, and G. Controls in Pandemic Influenza Working. 2013. Effectiveness of vaccination with 23-valent pneumococcal polysaccharide vaccine in preventing hospitalization with laboratory confirmed influenza during the 2009-2010 and 2010-2011 seasons. *Human vaccines & immunotherapeutics* 9: 865-873.
252. Chang, Y. C., Y. J. Chou, J. Y. Liu, T. F. Yeh, and N. Huang. 2012. Additive benefits of pneumococcal and influenza vaccines among elderly persons aged 75 years or older in Taiwan--a representative population-based comparative study. *J Infect* 65: 231-238.
253. Furumoto, A., Y. Ohkusa, M. Chen, K. Kawakami, H. Masaki, Y. Sueyasu, T. Iwanaga, H. Aizawa, T. Nagatake, and K. Oishi. 2008. Additive effect of pneumococcal vaccine and influenza vaccine on acute exacerbation in patients with chronic lung disease. *Vaccine* 26: 4284-4289.
254. Katsura, H., Z. Piao, K. Iwatsuki-Horimoto, Y. Akeda, S. Watanabe, T. Horimoto, K. Oishi, and Y. Kawaoka. 2014. A bivalent vaccine based on a replication-incompetent influenza virus protects against Streptococcus pneumoniae and influenza virus infection. *Journal of virology* 88: 13410-13417.

255. Dodd, D. 2003. Benefits of combination vaccines: effective vaccination on a simplified schedule. *Am J Manag Care* 9: S6-12.
256. Skibinski, D. A., B. C. Baudner, M. Singh, and D. T. O'Hagan. 2011. Combination vaccines. *J Glob Infect Dis* 3: 63-72.
257. (FDA), F. a. D. A. U. 2015. Complete List of Vaccines Licensed for Immunization and Distribution in the US.
258. Edwards, K. M., B. D. Meade, M. D. Decker, G. F. Reed, M. B. Rennels, M. C. Steinhoff, E. L. Anderson, J. A. Englund, M. E. Pichichero, and M. A. Deloria. 1995. Comparison of 13 acellular pertussis vaccines: overview and serologic response. *Pediatrics* 96: 548-557.
259. Richards, L., D. M. Ferreira, E. N. Miyaji, P. W. Andrew, and A. Kadioglu. 2010. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology* 215: 251-263.
260. Ivanov, S., J. Fontaine, C. Paget, E. Macho Fernandez, L. Van Maele, J. Renneson, I. Maillet, N. M. Wolf, A. Rial, H. Leger, B. Ryffel, B. Frisch, J. A. Chabalgoity, J. C. Sirard, A. Benecke, C. Faveeuw, and F. Trottein. 2012. Key role for respiratory CD103(+) dendritic cells, IFN-gamma, and IL-17 in protection against Streptococcus pneumoniae infection in response to alpha-galactosylceramide. *The Journal of infectious diseases* 206: 723-734.
261. Weibel, R. E., A. J. Carlson, Jr., V. M. Villarejos, E. B. Buynak, A. A. McLean, and M. R. Hilleman. 1980. Clinical and laboratory studies of combined live measles, mumps, and rubella vaccines using the RA 27/3 rubella virus. *Proc Soc Exp Biol Med* 165: 323-326.
262. Pabst, H. F., D. W. Spady, M. M. Carson, H. T. Stelfox, J. A. Beeler, and M. P. Krezolek. 1997. Kinetics of immunologic responses after primary MMR vaccination. *Vaccine* 15: 10-14.
263. Marshall, G. S., G. L. Adams, M. L. Leonardi, M. Petrecz, S. A. Flores, A. L. Ngai, J. Xu, G. Liu, J. E. Stek, G. Foglia, and A. W. Lee. 2015. Immunogenicity, Safety, and Tolerability of a Hexavalent Vaccine in Infants. *Pediatrics* 136: e323-332.
264. McCormack, P. L. 2013. DTaP-IPV-Hep B-Hib vaccine (Hexaxim(R)) : a review of its use in primary and booster vaccination. *Paediatr Drugs* 15: 59-70.
265. Jabbal-Gill, I. 2010. Nasal vaccine innovation. *Journal of drug targeting* 18: 771-786.
266. Brandtzaeg, P. 2011. Immune functions of nasopharyngeal lymphoid tissue. *Adv Otorhinolaryngol* 72: 20-24.
267. Kunisawa, J., T. Nochi, and H. Kiyono. 2008. Immunological commonalities and distinctions between airway and digestive immunity. *Trends in immunology* 29: 505-513.
268. Zuercher, A. W., S. E. Coffin, M. C. Thurnheer, P. Fundova, and J. J. Cebra. 2002. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *Journal of immunology* 168: 1796-1803.
269. Hellings, P., M. Jorissen, and J. L. Ceuppens. 2000. The Waldeyer's ring. *Acta Otorhinolaryngol Belg* 54: 237-241.
270. Fujimura, Y. 2000. Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans. *Virchows Arch* 436: 560-566.
271. Ogasawara, N., T. Kojima, M. Go, K. Takano, R. Kamekura, T. Ohkuni, J. Koizumi, T. Masaki, J. Fuchimoto, K. Obata, M. Kurose, T. Shintani, N. Sawada, and T. Himi. 2011. Epithelial barrier and antigen uptake in lymphoepithelium of human adenoids. *Acta Otolaryngol* 131: 116-123.
272. Shimoda, M., T. Nakamura, Y. Takahashi, H. Asanuma, S. Tamura, T. Kurata, T. Mizuochi, N. Azuma, C. Kanno, and T. Takemori. 2001. Isotype-specific selection of

high affinity memory B cells in nasal-associated lymphoid tissue. *The Journal of experimental medicine* 194: 1597-1607.
273. Zaman, M., S. Chandrudu, and I. Toth. 2013. Strategies for intranasal delivery of vaccines. *Drug Deliv Transl Res* 3: 100-109.
274. Dunne, P. J., B. Moran, R. C. Cummins, and K. H. Mills. 2009. CD11c+CD8alpha+ dendritic cells promote protective immunity to respiratory infection with Bordetella pertussis. *Journal of immunology* 183: 400-410.
275. McGhee, J. R., and K. Fujihashi. 2012. Inside the mucosal immune system. *PLoS Biol* 10: e1001397.
276. Williams, N. A., T. R. Hirst, and T. O. Nashar. 1999. Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic. *Immunol Today* 20: 95-101.
277. Spangler, B. D. 1992. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. *Microbiol Rev* 56: 622-647.
278. Lycke, N., and W. Strober. 1989. Cholera toxin promotes B cell isotype differentiation. *Journal of immunology* 142: 3781-3787.
279. Gizurarson, S., S. Tamura, C. Aizawa, and T. Kurata. 1992. Stimulation of the transepithelial flux of influenza HA vaccine by cholera toxin B subunit. *Vaccine* 10: 101-106.
280. Cong, Y., C. T. Weaver, and C. O. Elson. 1997. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. *Journal of immunology* 159: 5301-5308.
281. Gagliardi, M. C., F. Sallusto, M. Marinaro, A. Langenkamp, A. Lanzavecchia, and M. T. De Magistris. 2000. Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming. *European journal of immunology* 30: 2394-2403.
282. Marinaro, M., H. F. Staats, T. Hiroi, R. J. Jackson, M. Coste, P. N. Boyaka, N. Okahashi, M. Yamamoto, H. Kiyono, H. Bluethmann, K. Fujihashi, and J. R. McGhee. 1995. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J Immunol* 155: 4621-4629.
283. Lee, J. B., J. E. Jang, M. K. Song, and J. Chang. 2009. Intranasal delivery of cholera toxin induces th17-dominated T-cell response to bystander antigens. *PLoS One* 4: e5190.
284. Wu, H. Y., and M. W. Russell. 1993. Induction of mucosal immunity by intranasal application of a streptococcal surface protein antigen with the cholera toxin B subunit. *Infection and immunity* 61: 314-322.
285. Wu, H. Y., M. H. Nahm, Y. Guo, M. W. Russell, and D. E. Briles. 1997. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with Streptococcus pneumoniae. *The Journal of infectious diseases* 175: 839-846.
286. Xu, X., H. Wang, Y. Liu, Y. Wang, L. Zeng, K. Wu, J. Wang, F. Ma, W. Xu, Y. Yin, and X. Zhang. 2015. Mucosal immunization with the live attenuated vaccine SPY1 induces humoral and Th2-Th17-regulatory T cell cellular immunity and protects against pneumococcal infection. *Infection and immunity* 83: 90-100.
287. van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee. 2000. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J Immunol* 165: 4778-4782.
288. Lewis, D. J., Z. Huo, S. Barnett, I. Kromann, R. Giemza, E. Galiza, M. Woodrow, B. Thierry-Carstensen, P. Andersen, D. Novicki, G. Del Giudice, and R. Rappuoli. 2009. Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin. *PloS one* 4: e6999.

289. Coffman, R. L., A. Sher, and R. A. Seder. 2010. Vaccine adjuvants: putting innate immunity to work. *Immunity* 33: 492-503.
290. O'Neill, L. A., and A. G. Bowie. 2010. Sensing and signaling in antiviral innate immunity. *Curr Biol* 20: R328-333.
291. Haas, T., J. Metzger, F. Schmitz, A. Heit, T. Muller, E. Latz, and H. Wagner. 2008. The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. *Immunity* 28: 315-323.
292. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374: 546-549.
293. Jahrsdorfer, B., G. Hartmann, E. Racila, W. Jackson, L. Muhlenhoff, G. Meinhardt, S. Endres, B. K. Link, A. M. Krieg, and G. J. Weiner. 2001. CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. *Journal of leukocyte biology* 69: 81-88.
294. Pesce, I., E. Monaci, A. Muzzi, E. Tritto, S. Tavarini, S. Nuti, E. De Gregorio, and A. Wack. 2010. Intranasal administration of CpG induces a rapid and transient cytokine response followed by dendritic and natural killer cell activation and recruitment in the mouse lung. *J Innate Immun* 2: 144-159.
295. Lipford, G. B., M. Bauer, C. Blank, R. Reiter, H. Wagner, and K. Heeg. 1997. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *European journal of immunology* 27: 2340-2344.
296. Moldoveanu, Z., L. Love-Homan, W. Q. Huang, and A. M. Krieg. 1998. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* 16: 1216-1224.
297. Harper, D. M. 2009. Currently approved prophylactic HPV vaccines. *Expert Rev Vaccines* 8: 1663-1679.
298. Alving, C. R., K. K. Peachman, M. Rao, and S. G. Reed. 2012. Adjuvants for human vaccines. *Current opinion in immunology* 24: 310-315.
299. Ulrich, J. T., and K. R. Myers. 1995. Monophosphoryl lipid A as an adjuvant. In *Vaccine Design*. Springer. 495-524.
300. Baldridge, J. R., Y. Yorgensen, J. R. Ward, and J. T. Ulrich. 2000. Monophosphoryl lipid A enhances mucosal and systemic immunity to vaccine antigens following intranasal administration. *Vaccine* 18: 2416-2425.
301. Gorden, K. B., K. S. Gorski, S. J. Gibson, R. M. Kedl, W. C. Kieper, X. Qiu, M. A. Tomai, S. S. Alkan, and J. P. Vasilakos. 2005. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J Immunol* 174: 1259-1268.
302. Velasquez, L. S., B. E. Hjelm, C. J. Arntzen, and M. M. Herbst-Kralovetz. 2010. An intranasally delivered Toll-like receptor 7 agonist elicits robust systemic and mucosal responses to Norwalk virus-like particles. *Clin Vaccine Immunol* 17: 1850-1858.
303. Schroeder, H. W., Jr., and L. Cavacini. 2010. Structure and function of immunoglobulins. *J Allergy Clin Immunol* 125: S41-52.
304. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278: 1626-1629.
305. Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annual review of immunology* 15: 535-562.

306. Chen, H., and W. E. Paul. 1997. Cultured NK1.1+ CD4+ T cells produce large amounts of IL-4 and IFN-gamma upon activation by anti-CD3 or CD1. *Journal of immunology* 159: 2240-2249.
307. Singh, N., S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *Journal of immunology* 163: 2373-2377.
308. Cui, J., N. Watanabe, T. Kawano, M. Yamashita, T. Kamata, C. Shimizu, M. Kimura, E. Shimizu, J. Koike, H. Koseki, Y. Tanaka, M. Taniguchi, and T. Nakayama. 1999. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Valpha14 natural killer T cells. *The Journal of experimental medicine* 190: 783-792.
309. Carnaud, C., D. Lee, O. Donnars, S. H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *Journal of immunology* 163: 4647-4650.
310. Nishimura, T., H. Kitamura, K. Iwakabe, T. Yahata, A. Ohta, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, M. Taniguchi, M. Nakui, M. Sekimoto, and T. Koda. 2000. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *International immunology* 12: 987-994.
311. Park, S. H., and A. Bendelac. 2000. CD1-restricted T-cell responses and microbial infection. *Nature* 406: 788-792.
312. Galli, G., S. Nuti, S. Tavarini, L. Galli-Stampino, C. De Lalla, G. Casorati, P. Dellabona, and S. Abrignani. 2003. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *The Journal of experimental medicine* 197: 1051-1057.
313. Ko, S. Y., H. J. Ko, W. S. Chang, S. H. Park, M. N. Kweon, and C. Y. Kang. 2005. alpha-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *Journal of immunology* 175: 3309-3317.
314. Kamijuku, H., Y. Nagata, X. Jiang, T. Ichinohe, T. Tashiro, K. Mori, M. Taniguchi, K. Hase, H. Ohno, T. Shimaoka, S. Yonehara, T. Odagiri, M. Tashiro, T. Sata, H. Hasegawa, and K. I. Seino. 2008. Mechanism of NKT cell activation by intranasal coadministration of alpha-galactosylceramide, which can induce cross-protection against influenza viruses. *Mucosal Immunol* 1: 208-218.
315. Lowy, R. J., G. A. Vavrina, and D. D. LaBarre. 2001. Comparison of gamma and neutron radiation inactivation of influenza A virus. *Antiviral Res* 52: 261-273.
316. Sommers, C. H., and K. T. Rajkowski. 2011. Radiation inactivation of foodborne pathogens on frozen seafood products. *J Food Prot* 74: 641-644.
317. Marciniec, B., M. Stawny, M. Kozak, and M. Naskrent. 2008. The influence of radiation sterilization on thiamphenicol. *Spectrochim Acta A Mol Biomol Spectrosc* 69: 865-870.
318. Elliott, L. H., J. B. McCormick, and K. M. Johnson. 1982. Inactivation of Lassa, Marburg, and Ebola viruses by gamma irradiation. *J Clin Microbiol* 16: 704-708.
319. Grieb, T., R. Y. Forng, R. Brown, T. Owolabi, E. Maddox, A. McBain, W. N. Drohan, D. M. Mann, and W. H. Burgess. 2002. Effective use of gamma irradiation for pathogen inactivation of monoclonal antibody preparations. *Biologicals* 30: 207-216.

320. Sommer, R., W. Pribil, S. Appelt, P. Gehringer, H. Eschweiler, H. Leth, A. Cabaj, and T. Haider. 2001. Inactivation of bacteriophages in water by means of non-ionizing (UV-253.7 nm) and ionizing (gamma) radiation: a comparative approach. *Water Res* 35: 3109-3116.
321. Jordan, R. T., and L. L. Kempe. 1956. Inactivation of some animal viruses with gamma radiation from cobalt-60. *Proc Soc Exp Biol Med* 91: 212-215.
322. Sullivan, R., A. C. Fassolitis, E. P. Larkin, R. B. Read, Jr., and J. T. Peeler. 1971. Inactivation of thirty viruses by gamma radiation. *Appl Microbiol* 22: 61-65.
323. Osborne, J. C., Jr., J. H. Miller, and E. S. Kempner. 2000. Molecular mass and volume in radiation target theory. *Biophys J* 78: 1698-1702.
324. De Roda Husman, A. M., P. Bijkerk, W. Lodder, H. Van Den Berg, W. Pribil, A. Cabaj, P. Gehringer, R. Sommer, and E. Duizer. 2004. Calicivirus inactivation by nonionizing (253.7-nanometer-wavelength [UV]) and ionizing (gamma) radiation. *Appl Environ Microbiol* 70: 5089-5093.
325. Patterson, M. 1993. Food irradiation and food safety. *Reviews in Medical Microbiology* 4: 151-158.
326. Monk, J. D., L. R. Beuchat, and M. P. Doyle. 1995. Irradiation inactivation of food-borne microorganisms. *Journal of Food Protection¬Æ* 58: 197-208.
327. Reitman, M., H. R. Tribble, Jr., and L. Green. 1970. Gamma-irradiated Venezuelan equine encephalitis vaccines. *Appl Microbiol* 19: 763-767.
328. Geisbert, T. W., P. Pushko, K. Anderson, J. Smith, K. J. Davis, and P. B. Jahrling. 2002. Evaluation in nonhuman primates of vaccines against Ebola virus. *Emerging infectious diseases* 8: 503-507.
329. Furuya, Y., M. Regner, M. Lobigs, A. Koskinen, A. Mullbacher, and M. Alsharifi. 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.
330. Furuya, Y., J. Chan, E. C. Wan, A. Koskinen, K. R. Diener, J. D. Hayball, M. Regner, A. Mullbacher, and M. Alsharifi. 2011. Gamma-Irradiated Influenza Virus Uniquely Induces IFN-I Mediated Lymphocyte Activation Independent of the TLR7/MyD88 Pathway. *PLoS One* 6: e25765.
331. Alsharifi, M., M. Regner, R. Blanden, M. Lobigs, E. Lee, A. Koskinen, and A. Mullbacher. 2006. Exhaustion of type I interferon response following an acute viral infection. *J Immunol* 177: 3235-3241.
332. Wiktor, T. J., H. G. Aaslestad, and M. M. Kaplan. 1972. Immunogenicity of rabies virus inactivated by -propiolactone, acetylethyleneimine, and ionizing irradiation. *Appl Microbiol* 23: 914-918.
333. Matsevich, G. R., and I. A. Svet-Moldavskaia. 1970. [Smallpox vaccine inactivated by gamma-rays: its antigenic and immunogenic properties]. *Vopr Virusol* 15: 316-322.
334. Eisenberg, G. H., Jr., and J. V. Osterman. 1979. Gamma-irradiated scrub typhus immunogens: broad-spectrum immunity with combinations of rickettsial strains. *Infection and immunity* 26: 131-136.
335. Datta, S. K., S. Okamoto, T. Hayashi, S. S. Shin, I. Mihajlov, A. Fermin, D. G. Guiney, J. Fierer, and E. Raz. 2006. Vaccination with irradiated Listeria induces protective T cell immunity. *Immunity* 25: 143-152.
336. Sanakkayala, N., A. Sokolovska, J. Gulani, H. Hogenesch, N. Sriranganathan, S. M. Boyle, G. G. Schurig, and R. Vemulapalli. 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant Brucella abortus RB51. *Clinical and diagnostic laboratory immunology* 12: 1429-1436.

337. Gordon, M., D. M. Donaldson, and G. G. Wright. 1964. Immunization of Mice with Irradiated Pasteurella Tularensis. *The Journal of infectious diseases* 114: 435-440.
338. Carpenter, C. M., A. W. Naylor-Foote, G. V. Taplin, C. A. Lawrence, and C. L. Drake. 1959. Preliminary report on vaccines prepared from gamma-irradiated Mycobacterium tuberculosis and Brucella suis. *Am Rev Tuberc* 79: 374-377.
339. Howard-Flanders, P. 1961. Factors affecting radiation injury to DNA in bacteria and bacteriophage systems. *Brookhaven Symp Biol* 14: 18-31.
340. Glendenning, O. M., and F. J. Roe. 1956. The carcinogenicity of beta-propiolactone for mouseskin. *Br J Cancer* 10: 357-362.
341. Hueper, W. C. 1963. Environmental Carcinogenesis in Man and Animals. *Annals of the New York Academy of Sciences* 108: 963-1038.
342. Hiramoto, R. M., A. J. Galisteo, N. do Nascimento, and H. F. de Andrade, Jr. 2002. 200 Gy sterilised Toxoplasma gondii tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice. *Vaccine* 20: 2072-2081.
343. Chen, A., B. Mann, G. Gao, R. Heath, J. King, J. Maissoneuve, M. Alderson, A. Tate, S. K. Hollingshead, R. K. Tweten, D. E. Briles, E. I. Tuomanen, and J. C. Paton. 2015. Multivalent Pneumococcal Protein Vaccines Comprising Pneumolysoid with Epitopes/Fragments of CbpA and/or PspA Elicit Strong and Broad Protection. *Clin Vaccine Immunol*.
344. Cottey, R., C. A. Rowe, and B. S. Bender. 2001. Influenza virus. *Curr Protoc Immunol* Chapter 19: Unit 19 11.
345. Harvey, R. M., A. D. Ogunniyi, A. Y. Chen, and J. C. Paton. 2011. Pneumolysin with low hemolytic activity confers an early growth advantage to Streptococcus pneumoniae in the blood. *Infection and immunity* 79: 4122-4130.
346. Horton, R. M., S. N. Ho, J. K. Pullen, H. D. Hunt, Z. Cai, and L. R. Pease. 1993. Gene splicing by overlap extension. *Methods Enzymol* 217: 270-279.
347. Berry, A. M., A. D. Ogunniyi, D. C. Miller, and J. C. Paton. 1999. Comparative virulence of Streptococcus pneumoniae strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infection and immunity* 67: 981-985.
348. Babb, R., J. Chan, J. E. Khairat, Y. Furuya, and M. Alsharifi. 2014. Gamma-Irradiated Influenza A Virus Provides Adjuvant Activity to a Co-Administered Poorly Immunogenic SFV Vaccine in Mice. *Front Immunol* 5: 267.
349. Paton, J. C., R. A. Lock, and D. J. Hansman. 1983. Effect of immunization with pneumolysin on survival time of mice challenged with Streptococcus pneumoniae. *Infection and immunity* 40: 548-552.
350. Trappetti, C., E. van der Maten, Z. Amin, A. J. Potter, A. Y. Chen, P. M. van Mourik, A. J. Lawrence, A. W. Paton, and J. C. Paton. 2013. Site of isolation determines biofilm formation and virulence phenotypes of Streptococcus pneumoniae serotype 3 clinical isolates. *Infection and immunity* 81: 505-513.
351. Montgomery, C. P., M. Daniels, F. Zhao, M. L. Alegre, A. S. Chong, and R. S. Daum. 2014. Protective immunity against recurrent Staphylococcus aureus skin infection requires antibody and interleukin-17A. *Infection and immunity* 82: 2125-2134.
352. Haylock-Jacobs, S., I. Comerford, M. Bunting, E. Kara, S. Townley, M. Klingler-Hoffmann, B. Vanhaesebroeck, K. D. Puri, and S. R. McColl. 2011. PI3Kdelta drives the pathogenesis of experimental autoimmune encephalomyelitis by inhibiting effector T cell apoptosis and promoting Th17 differentiation. *Journal of autoimmunity* 36: 278-287.

353. Malley, R., and P. W. Anderson. 2012. Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity. *Proceedings of the National Academy of Sciences of the United States of America* 109: 3623-3627.
354. Liu, Y., H. Wang, S. Zhang, L. Zeng, X. Xu, K. Wu, W. Wang, N. Yin, Z. Song, X. Zhang, and Y. Yin. 2014. Mucosal immunization with recombinant fusion protein DnaJ-DeltaA146Ply enhances cross-protective immunity against Streptococcus pneumoniae infection in mice via interleukin 17A. *Infection and immunity* 82: 1666-1675.
355. Basset, A., C. M. Thompson, S. K. Hollingshead, D. E. Briles, E. W. Ades, M. Lipsitch, and R. Malley. 2007. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infection and immunity* 75: 5460-5464.
356. Ingram, L. O. 1990. Ethanol tolerance in bacteria. *Critical reviews in biotechnology* 9: 305-319.
357. Brundage, J. F. 2006. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. *The Lancet. Infectious diseases* 6: 303-312.
358. Nims, R. W., G. Gauvin, and M. Plavsic. 2011. Gamma irradiation of animal sera for inactivation of viruses and mollicutes--a review. *Biologicals* 39: 370-377.
359. Bosch, A. A., G. Biesbroek, K. Trzcinski, E. A. Sanders, and D. Bogaert. 2013. Viral and bacterial interactions in the upper respiratory tract. *PLoS pathogens* 9: e1003057.
360. Sun, K., S. L. Salmon, S. A. Lotz, and D. W. Metzger. 2007. Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory Streptococcus pneumoniae infection. *Infection and immunity* 75: 1196-1202.
361. Yamada, M., J. C. Gomez, P. E. Chugh, C. A. Lowell, M. C. Dinauer, D. P. Dittmer, and C. M. Doerschuk. 2011. Interferon-gamma production by neutrophils during bacterial pneumonia in mice. *Am J Respir Crit Care Med* 183: 1391-1401.
362. Shin, H., and A. Iwasaki. 2013. Tissue-resident memory T cells. *Immunological reviews* 255: 165-181.
363. Hogan, R. J., W. Zhong, E. J. Usherwood, T. Cookenham, A. D. Roberts, and D. L. Woodland. 2001. Protection from respiratory virus infections can be mediated by antigen-specific CD4(+) T cells that persist in the lungs. *The Journal of experimental medicine* 193: 981-986.
364. Michel, M. L., A. C. Keller, C. Paget, M. Fujio, F. Trottein, P. B. Savage, C. H. Wong, E. Schneider, M. Dy, and M. C. Leite-de-Moraes. 2007. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *The Journal of experimental medicine* 204: 995-1001.
365. Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure. 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infection and immunity* 62: 2582-2589.
366. Weiser, J. N., Z. Markiewicz, E. I. Tuomanen, and J. H. Wani. 1996. Relationship between phase variation in colony morphology, intrastrain variation in cell wall physiology, and nasopharyngeal colonization by Streptococcus pneumoniae. *Infection and immunity* 64: 2240-2245.
367. McCool, T. L., and J. N. Weiser. 2004. Limited role of antibody in clearance of Streptococcus pneumoniae in a murine model of colonization. *Infection and immunity* 72: 5807-5813.

368. Lipsitch, M., C. G. Whitney, E. Zell, T. Kaijalainen, R. Dagan, and R. Malley. 2005. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? *PLoS Med* 2: e15.
369. Robinson, K. M., J. K. Kolls, and J. F. Alcorn. 2015. The immunology of influenza virus-associated bacterial pneumonia. *Current opinion in immunology* 34: 59-67.
370. Kudva, A., E. V. Scheller, K. M. Robinson, C. R. Crowe, S. M. Choi, S. R. Slight, S. A. Khader, P. J. Dubin, R. I. Enelow, J. K. Kolls, and J. F. Alcorn. 2011. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *Journal of Immunology* 186: 1666-1674.
371. Kadioglu, A., A. M. Cuppone, C. Trappetti, T. List, A. Spreafico, G. Pozzi, P. W. Andrew, and M. R. Oggioni. 2011. Sex-based differences in susceptibility to respiratory and systemic pneumococcal disease in mice. *The Journal of infectious diseases* 204: 1971-1979.
372. Kao, C. Y., Y. Chen, P. Thai, S. Wachi, F. Huang, C. Kim, R. W. Harper, and R. Wu. 2004. IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *Journal of Immunology* 173: 3482-3491.
373. Lee, H. Y., A. Andalibi, P. Webster, S. K. Moon, K. Teufert, S. H. Kang, J. D. Li, M. Nagura, T. Ganz, and D. J. Lim. 2004. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable Haemophilus influenzae. *BMC Infect Dis* 4: 12.
374. van Gils, E. J., E. Hak, R. H. Veenhoven, G. D. Rodenburg, D. Bogaert, J. P. Bruin, L. van Alphen, and E. A. Sanders. 2011. Effect of seven-valent pneumococcal conjugate vaccine on Staphylococcus aureus colonisation in a randomised controlled trial. *PLoS One* 6: e20229.
375. Jabbal-Gill, I., P. Watts, and A. Smith. 2012. Chitosan-based delivery systems for mucosal vaccines. *Expert opinion on drug delivery* 9: 1051-1067.
376. Smith, A., M. Perelman, and M. Hinchcliffe. 2014. Chitosan: a promising safe and immune-enhancing adjuvant for intranasal vaccines. *Human vaccines & immunotherapeutics* 10: 797-807.
377. Bromander, A., J. Holmgren, and N. Lycke. 1991. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. *Journal of immunology* 146: 2908-2914.
378. Fujihashi, K., T. Koga, F. W. van Ginkel, Y. Hagiwara, and J. R. McGhee. 2002. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. *Vaccine* 20: 2431-2438.
379. Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *The New England journal of medicine* 350: 896-903.
380. Atmar, R. L., D. I. Bernstein, C. D. Harro, M. S. Al-Ibrahim, W. H. Chen, J. Ferreira, M. K. Estes, D. Y. Graham, A. R. Opekun, C. Richardson, and P. M. Mendelman. 2011. Norovirus vaccine against experimental human Norwalk Virus illness. *The New England journal of medicine* 365: 2178-2187.
381. El-Kamary, S. S., M. F. Pasetti, P. M. Mendelman, S. E. Frey, D. I. Bernstein, J. J. Treanor, J. Ferreira, W. H. Chen, R. Sublett, C. Richardson, R. F. Bargatze, M. B. Sztein, and C. O. Tacket. 2010. Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues. *J Infect Dis* 202: 1649-1658.

382. Read, R. C., S. C. Naylor, C. W. Potter, J. Bond, I. Jabbal-Gill, A. Fisher, L. Illum, and R. Jennings. 2005. Effective nasal influenza vaccine delivery using chitosan. *Vaccine* 23: 4367-4374.
383. McNeela, E. A., D. O'Connor, I. Jabbal-Gill, L. Illum, S. S. Davis, M. Pizza, S. Peppoloni, R. Rappuoli, and K. H. Mills. 2000. A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM(197)) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery. *Vaccine* 19: 1188-1198.
384. Morris, G., S. Kok, S. Harding, and G. Adams. 2010. Polysaccharide drug delivery systems based on pectin and chitosan. *Biotechnology & genetic engineering reviews* 27: 257-284.
385. Illum, L., I. Jabbal-Gill, M. Hinchcliffe, A. N. Fisher, and S. S. Davis. 2001. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev* 51: 81-96.
386. Dodane, V., M. Amin Khan, and J. R. Merwin. 1999. Effect of chitosan on epithelial permeability and structure. *Int J Pharm* 182: 21-32.
387. Jabbal-Gill, I., A. N. Fisher, R. Rappuoli, S. S. Davis, and L. Illum. 1998. Stimulation of mucosal and systemic antibody responses against Bordetella pertussis filamentous haemagglutinin and recombinant pertussis toxin after nasal administration with chitosan in mice. *Vaccine* 16: 2039-2046.
388. Boonyo, W., H. E. Junginger, N. Waranuch, A. Polnok, and T. Pitaksuteepong. 2007. Chitosan and trimethyl chitosan chloride (TMC) as adjuvants for inducing immune responses to ovalbumin in mice following nasal administration. *J Control Release* 121: 168-175.
389. Svindland, S. C., G. K. Pedersen, R. D. Pathirana, G. Bredholt, J. K. Nostbakken, A. Jul-Larsen, C. A. Guzman, E. Montomoli, G. Lapini, S. Piccirella, I. Jabbal-Gill, M. Hinchcliffe, and R. J. Cox. 2013. A study of Chitosan and c-di-GMP as mucosal adjuvants for intranasal influenza H5N1 vaccine. *Influenza Other Respir Viruses* 7: 1181-1193.
390. McNeela, E. A., I. Jabbal-Gill, L. Illum, M. Pizza, R. Rappuoli, A. Podda, D. J. Lewis, and K. H. Mills. 2004. Intranasal immunization with genetically detoxified diphtheria toxin induces T cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan. *Vaccine* 22: 909-914.
391. Mori, A., E. Oleszycka, F. A. Sharp, M. Coleman, Y. Ozasa, M. Singh, D. T. O'Hagan, L. Tajber, O. I. Corrigan, E. A. McNeela, and E. C. Lavelle. 2012. The vaccine adjuvant alum inhibits IL-12 by promoting PI3 kinase signaling while chitosan does not inhibit IL-12 and enhances Th1 and Th17 responses. *European journal of immunology* 42: 2709-2719.
392. Wen, Z. S., Y. L. Xu, X. T. Zou, and Z. R. Xu. 2011. Chitosan nanoparticles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. *Mar Drugs* 9: 1038-1055.
393. Xu, J., W. Dai, Z. Wang, B. Chen, Z. Li, and X. Fan. 2011. Intranasal vaccination with chitosan-DNA nanoparticles expressing pneumococcal surface antigen a protects mice against nasopharyngeal colonization by Streptococcus pneumoniae. *Clin Vaccine Immunol* 18: 75-81.
394. Meza-Sanchez, D., G. Perez-Montesinos, J. Sanchez-Garcia, J. Moreno, and L. C. Bonifaz. 2011. Intradermal immunization in the ear with cholera toxin and its non-toxic beta subunit promotes efficient Th1 and Th17 differentiation dependent on migrating DCs. *European journal of immunology* 41: 2894-2904.
395. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells

regulates tissue inflammation by producing interleukin 17. *Nature immunology* 6: 1133-1141.
396. Yu, J. J., and S. L. Gaffen. 2008. Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity. *Front Biosci* 13: 170-177.
397. Jaffar, Z., M. E. Ferrini, L. A. Herritt, and K. Roberts. 2009. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. *Journal of immunology* 182: 4507-4511.
398. Casey, K. A., K. A. Fraser, J. M. Schenkel, A. Moran, M. C. Abt, L. K. Beura, P. J. Lucas, D. Artis, E. J. Wherry, K. Hogquist, V. Vezys, and D. Masopust. 2012. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *Journal of immunology* 188: 4866-4875.
399. Jackson, R. J., K. Fujihashi, J. Xu-Amano, H. Kiyono, C. O. Elson, and J. R. McGhee. 1993. Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. *Infection and immunity* 61: 4272-4279.
400. Kobayashi, T., K. Fukushima, T. Sannan, N. Saito, Y. Takiguchi, Y. Sato, H. Hasegawa, and K. Ishikawa. 2013. Evaluation of the effectiveness and safety of chitosan derivatives as adjuvants for intranasal vaccines. *Viral Immunol* 26: 133-142.
401. Soane, R. J., M. Frier, A. C. Perkins, N. S. Jones, S. S. Davis, and L. Illum. 1999. Evaluation of the clearance characteristics of bioadhesive systems in humans. *Int J Pharm* 178: 55-65.
402. Kim, T. H., J. W. Nah, M. H. Cho, T. G. Park, and C. S. Cho. 2006. Receptor-mediated gene delivery into antigen presenting cells using mannosylated chitosan/DNA nanoparticles. *J Nanosci Nanotechnol* 6: 2796-2803.
403. Stahl, P. D., and R. A. Ezekowitz. 1998. The mannose receptor is a pattern recognition receptor involved in host defense. *Current opinion in immunology* 10: 50-55.
404. Egan, B. S., R. Abdolrasulnia, and V. L. Shepherd. 2004. IL-4 modulates transcriptional control of the mannose receptor in mouse FSDC dendritic cells. *Archives of biochemistry and biophysics* 428: 119-130.
405. Ding, J., L. Na, and S. Mao. 2012. Chitosan and its derivatives as the carrier for intranasal drug delivery. *Asian J Pharm Sci* 7: 349-361.
406. Hagenaars, N., E. Mastrobattista, R. J. Verheul, I. Mooren, H. L. Glansbeek, J. G. Heldens, H. van den Bosch, and W. Jiskoot. 2009. Physicochemical and immunological characterization of N,N,N-trimethyl chitosan-coated whole inactivated influenza virus vaccine for intranasal administration. *Pharm Res* 26: 1353-1364.
407. Gilchrist, S. A., A. Nanni, and O. Levine. 2012. Benefits and effectiveness of administering pneumococcal polysaccharide vaccine with seasonal influenza vaccine: an approach for policymakers. *Am J Public Health* 102: 596-605.
408. Halsey, N. A. 2001. Safety of combination vaccines: perception versus reality. *Pediatr Infect Dis J* 20: S40-44.
409. Kara, E. E., I. Comerford, K. A. Fenix, C. R. Bastow, C. E. Gregor, D. R. McKenzie, and S. R. McColl. 2014. Tailored immune responses: novel effector helper T cell subsets in protective immunity. *PLoS pathogens* 10: e1003905.
410. Park, C. O., and T. S. Kupper. 2015. The emerging role of resident memory T cells in protective immunity and inflammatory disease. *Nature medicine* 21: 688-697.
411. Hogan, R. J., E. J. Usherwood, W. Zhong, A. A. Roberts, R. W. Dutton, A. G. Harmsen, and D. L. Woodland. 2001. Activated antigen-specific CD8+ T cells persist

in the lungs following recovery from respiratory virus infections. *Journal of Immunology* 166: 1813-1822.
412. Nakamura, S., K. M. Davis, and J. N. Weiser. 2011. Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice. *The Journal of clinical investigation* 121: 3657-3665.
413. Sathaliyawala, T., M. Kubota, N. Yudanin, D. Turner, P. Camp, J. J. Thome, K. L. Bickham, H. Lerner, M. Goldstein, M. Sykes, T. Kato, and D. L. Farber. 2013. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* 38: 187-197.
414. Feldman, C., and R. Anderson. 2014. Review: current and new generation pneumococcal vaccines. *J Infect* 69: 309-325.
415. LeMessurier, K. S., H. Hacker, L. Chi, E. Tuomanen, and V. Redecke. 2013. Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung. *PLoS pathogens* 9: e1003727.
416. Imai, Y., K. Kuba, G. G. Neely, R. Yaghubian-Malhami, T. Perkmann, G. van Loo, M. Ermolaeva, R. Veldhuizen, Y. H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J. S. Peiris, A. S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C. J. Binder, and J. M. Penninger. 2008. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* 133: 235-249.
417. Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179-189.
418. Higgins, S. C., A. G. Jarnicki, E. C. Lavelle, and K. H. Mills. 2006. TLR4 mediates vaccine-induced protective cellular immunity to Bordetella pertussis: role of IL-17-producing T cells. *Journal of Immunology* 177: 7980-7989.
419. Maroof, A., Y. M. Yorgensen, Y. Li, and J. T. Evans. 2014. Intranasal vaccination promotes detrimental Th17-mediated immunity against influenza infection. *PLoS pathogens* 10: e1003875.
420. McKinstry, K. K., T. M. Strutt, A. Buck, J. D. Curtis, J. P. Dibble, G. Huston, M. Tighe, H. Hamada, S. Sell, R. W. Dutton, and S. L. Swain. 2009. IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge. *Journal of Immunology* 182: 7353-7363.
421. Crowe, C. R., K. Chen, D. A. Pociask, J. F. Alcorn, C. Krivich, R. I. Enelow, T. M. Ross, J. L. Witztum, and J. K. Kolls. 2009. Critical role of IL-17RA in immunopathology of influenza infection. *Journal of Immunology* 183: 5301-5310.
422. Lin, L., A. S. Ibrahim, X. Xu, J. M. Farber, V. Avanesian, B. Baquir, Y. Fu, S. W. French, J. E. Edwards, Jr., and B. Spellberg. 2009. Th1-Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in mice. *PLoS pathogens* 5: e1000703.
423. Goodwin, K., C. Viboud, and L. Simonsen. 2006. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine* 24: 1159-1169.
424. Lal, H., A. L. Cunningham, and T. C. Heineman. 2015. Adjuvanted Herpes Zoster Subunit Vaccine in Older Adults. *The New England journal of medicine* 373: 1576-1577.

APPENDIX

Appendix:

- Statement of Authorship forms (Chapter 3, 4 & 5)
- Published manuscript:

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Statement of Authorship

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Contribution to the Paper	Designed, performed and analysed experiments Wrote the manuscript		
Overall percentage (%)	80%		
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