

# Enhanced protective responses to a serotype-independent pneumococcal vaccine when combined with an inactivated influenza vaccine

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## Abstract

*Streptococcus pneumoniae* and influenza are the world's foremost bacterial and viral respiratory pathogens. We have previously described a  $\gamma$ -irradiated influenza A virus ( $\gamma$ -FLU) vaccine that provides cross-protective immunity against heterosubtypic infections. More recently, we reported a novel non-adjuvanted  $\gamma$ -irradiated *S. pneumoniae* ( $\gamma$ -PN) vaccine that elicits serotype-independent protection. Considering the clinical synergism of both pathogens, combination of a serotype-independent pneumococcal vaccine with a broad-spectrum influenza vaccine to protect against both infections would have a considerable clinical impact. In the present study, we co-immunized C57BL/6 mice intranasally (IN) with a mixture of  $\gamma$ -PN (whole inactivated cells) and  $\gamma$ -FLU (whole inactivated virions) and examined protective efficacy. Co-immunization enhanced  $\gamma$ -PN vaccine efficacy against virulent pneumococcal challenge, which was dependent on CD4<sup>+</sup> T-cell responses. In contrast, vaccination with  $\gamma$ -PN alone, co-immunization enhanced pneumococcal-specific effector T-helper 17 cell (Th17) and Th1 memory cell, promoted development of CD4<sup>+</sup> tissue-resident memory (TRM) cells and enhanced *Pneumococcus*-specific antibody responses. Furthermore, co-immunization elicited significant protection against lethal influenza challenge, as well as against co-infection with both influenza and *S. pneumoniae*. This is the first report showing the synergistic effect of combining whole cell and whole virion vaccines to both *S. pneumoniae* and influenza as a single vaccine to protect against individual and co-infection, without compromising pathogen-specific immunity.

**Key words:** co-infection, combination vaccine, influenza A virus, protective immunity, *Streptococcus pneumoniae*.

## INTRODUCTION

*Streptococcus pneumoniae* is a leading cause of morbidity and mortality. Upon nasopharyngeal colonization, co-infection with other pathogens such as influenza can promote progression to disease, resulting in pathologies such as pneumonia, bacteraemia, meningitis and otitis media [1]. In addition, secondary bacterial infection by *S. pneumoniae* following initial influenza infection is specifically associated with high mortality rates, particularly during influenza pandemics [2]. Lethal synergism of these two pathogens results from 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 the host's susceptibility to pneumococcal infection [3,4]. Thus, vaccination against influenza and/or *S. pneumoniae* represents an essential control strategy against these 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 [5–10]. However, despite the success of single vaccination regimes, lack of effective

**Abbreviations:**  $\gamma$ -FLU,  $\gamma$ -irradiated influenza A virus; IL, interleukin; IN, intranasally; IP, intraperitoneally; OPL, oxidized phospholipid;  $\gamma$ -PN,  $\gamma$ -irradiated *S. pneumoniae*; SFV, Semliki Forest virus; TCID, tissue culture infective dose; Th17, T-helper 17 cell; TRM, tissue-resident memory.

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vaccines that offer broad-spectrum, serotype-independent and heterosubtypic protection against *S. pneumoniae* and influenza respectively, remains a significant problem [11,12].

Combination vaccines have been employed to induce protective immunity to several pathogens simultaneously, thereby minimizing the number of injections individuals have to experience. This in turn significantly simplifies routine childhood immunization regimes and compliance [13]. Importantly, the immune response induced by each component of the combination vaccines should not inhibit or interfere with the quality of immunity induced by individual antigens. To our knowledge, the possibility of combining a whole-cell killed bacterial vaccine and an inactivated whole virus vaccine in a single vaccination regime to overcome the synergism between influenza and *S. pneumoniae* infection has not been explored.

We previously demonstrated the effectiveness of  $\gamma$ -irradiation as an inactivation technique for development of separate influenza ( $\gamma$ -irradiated influenza A virus,  $\gamma$ -FLU) and  $\gamma$ -irradiated *S. pneumoniae* ( $\gamma$ -PN) vaccines [14–16]. Intranasal vaccination with  $\gamma$ -FLU induces heterosubtypic protection against lethal challenge. Similarly, intranasal vaccination with  $\gamma$ -PN elicits serotype-independent protection against *S. pneumoniae*, which is reliant on B-cell and innate-derived interleukin (IL)-17 responses. Furthermore,  $\gamma$ -FLU possesses adjuvant properties, since its addition to a poorly immunogenic  $\gamma$ -irradiated Semliki Forest virus (SFV) vaccine enhanced anti-SFV humoral responses [17]. Thus, we investigated whether intranasal co-immunization with  $\gamma$ -PN and  $\gamma$ -FLU ( $\gamma$ -PN +  $\gamma$ -FLU) could provide protection against each pathogen, as well as against the enhanced pathogenesis associated with co-infection. In the present study, we demonstrate that co-immunization enhanced protective efficacy of  $\gamma$ -PN against individual and co-infection with *S. pneumoniae* and influenza. Strikingly, in contrast with  $\gamma$ -PN vaccination alone, the combination vaccine triggered development of CD4<sup>+</sup> tissue-resident memory (TRM) cells and augmented *S. pneumoniae*-specific T-helper 17 cell (Th17) and Th1 memory cell responses. This represents the first report demonstrating the protective efficacy of a combination vaccine containing viral and bacterial inactivated pathogens, and the enhanced effectiveness compared with individual administration of a pneumococcal vaccine.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*S. pneumoniae* strains have been recently described [18]. D39 (serotype 2) and EF3030 (serotype 19F) were grown statically at 37 °C in 5% CO<sub>2</sub> in serum broth (SB) to A<sub>600</sub> of 0.18, as described recently [18].

### Vaccines

*S. pneumoniae* strain, Rx1 [PdT/  $\Delta$ LytA] ( $4 \times 10^{10}$  CFU/ml) [16] and influenza strain, A/PR8 [ $3 \times 10^8$ , 50% tissue culture infective dose per ml (TCID<sub>50</sub>/ml)] [17] were grown and concentrated as described previously.

### Ethics

Animal experiments were approved by the University of Adelaide Animal Ethics Committee, Australia (approval numbers S-2010-001 and S-2013-053).

### Vaccination

C57BL/6 mice (5–6 weeks old) were supplied by Laboratory Animal Services, University of Adelaide, Australia. Mice were anaesthetized intraperitoneally (IP) with pentobarbital sodium (Nembutal; Ilium; 66 mg/g body weight). Anaesthetized mice were given  $\gamma$ -PN ( $\sim 10^8$  CFU-equivalent/dose) or  $\gamma$ -FLU ( $\sim 10^6$  TCID<sub>50</sub>-equivalent/dose) in 30  $\mu$ l intranasally (IN). For co-vaccination, the appropriate doses of each vaccine were combined in a final volume of 30  $\mu$ l. Mice were immunized IN twice at 2 weeks intervals.

### Infection models

#### Intranasal sepsis model

Mice were anaesthetized and challenged IN with 10<sup>6</sup> CFU of D39. Mice were monitored for up to 21 days or until moribund.

#### Intranasal colonization-induced pneumonia model

Unanaesthetized C57BL/6 mice were infected with EF3030 by applying 10  $\mu$ l containing 10<sup>7</sup> CFU to the nostrils to allow for bacterial colonization. Four days later, mice were anaesthetized and given 30  $\mu$ l of PBS IN to induce bacterial migration to the lung. Seven days later, mice were killed and the nasal wash, nasopharyngeal and lung tissue were harvested and plated for CFU recovery as described previously [19]. Total CFU in nasopharynx includes CFU recovery from nasal wash and nasal tissue combined. Another group of mice was monitored for survival for 21 days.

#### Intranasal colonization model

Mice were colonized with EF3030 and nasopharyngeal bacterial loads were analysed as described above.

#### Influenza challenge model

Mice were challenged IN with A/PR8 ( $\sim 200$  TCID<sub>50</sub>) and the weights of the mice were recorded for 21 days. Percentage weight loss was calculated from original weight before challenge. Mice were killed at 20% weight loss as per ethical guidelines.

#### Co-infection model

Mice were colonized with EF3030 as described above. Four days later, mice were anaesthetized and infected IN with a subclinical dose of A/PR8 (10 TCID<sub>50</sub>) and monitored for up to 21 days post co-infection.

### Measurement of antibody responses

Serum samples were collected 2 weeks after the second immunization and assayed by ELISA to determine *S. pneumoniae*-specific and A/PR8-specific antibody responses, as described recently [17,18]. For *S. pneumoniae*-specific antibody analysis, the whole-cell vaccine strain Rx1 [PdT/ $\Delta$ LytA] was grown and

concentrated as described recently [16], but was resuspended in PBS with 80% glycerol. ELISA plates were coated with 50  $\mu$ l/well of a  $10^8$  CFU/ml bacterial suspension diluted in bicarbonate coating buffer. Endpoint titres are expressed as the reciprocal of the last dilution whose absorbance value was greater than three S.D. above the mean of the absorbance values of samples from the negative control (PBS-treated) mice.

### Lymphocyte stimulation with the $\gamma$ -PN or $\gamma$ -FLU vaccine

Lung and spleen single cell suspensions were stimulated in culture with  $\gamma$ -PN or  $\gamma$ -FLU antigen as described recently [16].

### CD4 depletion *in vivo*

Mice were given four doses IP (150  $\mu$ g in 200  $\mu$ l of PBS/dose) of anti-CD4 (GK1.5) or anti-rat IgG2b (LTF-2) at day -2, -1, 1 and 4 (6 h before IN administration of PBS).

### Intracellular cytokine staining and flow cytometry

Lung cell suspensions obtained after D39 challenge were stimulated with  $\gamma$ -PN or  $\gamma$ -FLU vaccine antigens and assessed using intracellular cytokine staining as described previously [20]. Cells were permeabilized using Cytofix/Cytoperm kit (BD) according to the manufacturer's instructions. Surface antigens were stained using the following antibodies: anti-CD3 (BioLegend), anti-CD4 (eBioscience), anti-CD44 (eBioscience), anti-CD103 (eBioscience) and anti-CD11a (eBioscience). The following intracellular antibodies were used: anti-IL-17A (BioLegend), anti-IFN- $\gamma$  (eBioscience), anti-IL-4 (eBioscience) and anti-Foxp3 (eBioscience). Biotin-conjugated antibodies were detected using Streptavidin-BV450 (BD). Cells were acquired using LSR II flow cytometer (BD) and analysed using FlowJo software (TreeStar).

### Statistics

Data were analysed using a Fisher's exact test or a one-way ANOVA with appropriate post-tests as indicated in the text. Statistical analyses were performed using GraphPad Prism. *P*-values  $\leq 0.05$  were considered significant.

## RESULTS

### Co-immunization enhances the protective efficacy of the $\gamma$ -PN vaccine

We have previously demonstrated the protective efficacy of  $\gamma$ -FLU and  $\gamma$ -PN vaccines against lethal challenge [16,17,21]. In the present study, we investigated whether administering  $\gamma$ -PN +  $\gamma$ -FLU as a combined vaccine modulates protective efficacy of  $\gamma$ -PN. Mice were immunized IN with  $\gamma$ -FLU and/or  $\gamma$ -PN and challenged IN with *S. pneumoniae* D39. Immunization with  $\gamma$ -PN alone or co-administered with  $\gamma$ -FLU provided protection against lethal D39 challenge (Figure 1A). Co-vaccinated animals showed a slightly higher rate of survival compared with those vaccinated with  $\gamma$ -PN alone. As expected,  $\gamma$ -FLU immunization did not protect mice from lethal pneumococcal challenge.

Thus, co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU vaccines does not adversely affect the protective efficacy of  $\gamma$ -PN vaccination in a model of lethal pneumococcal sepsis. To further investigate the efficacy of the combination vaccine, mice were challenged with the colonization-induced pneumonia model. Co-immunized mice were the only group to show significant survival rates (Figure 1B) and a significant reduction in bacterial load in the nasopharynx and lungs (Figure 1C). Similarly, nasopharyngeal counts were also significantly reduced after challenge with the colonization model in co-immunized mice (Figure 1D). These data demonstrate that co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU does not compromise, but rather enhances the protective efficacy of  $\gamma$ -PN.

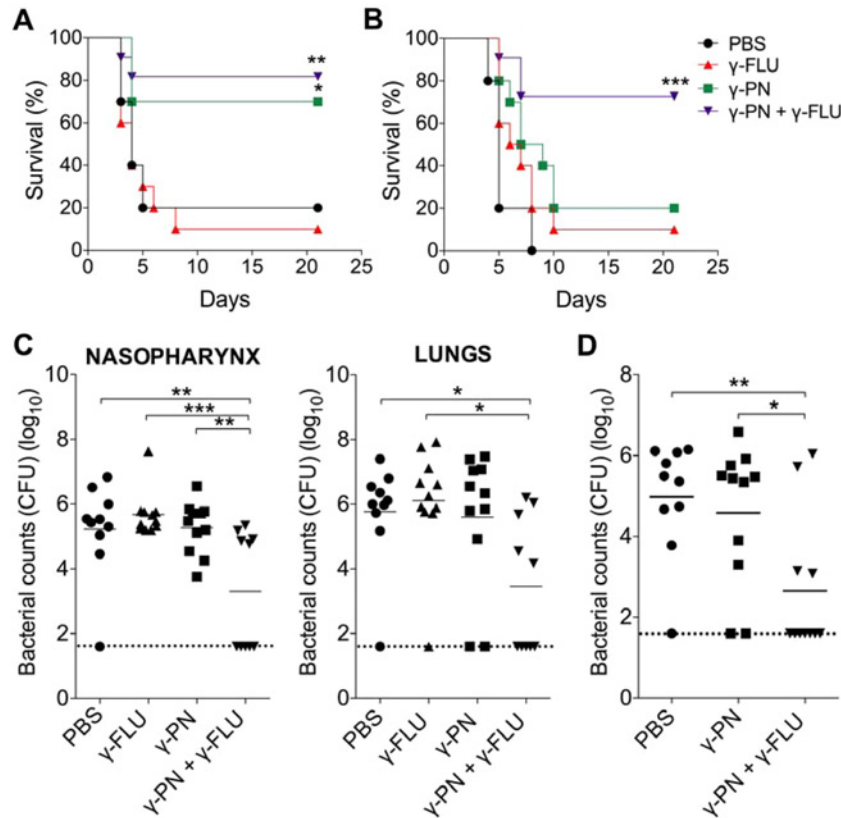
### Mechanism responsible for the enhanced protection elicited by co-immunization

To assess how co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU shapes pathogen-specific antibody responses, immune sera were analysed for *Pneumococcus*-specific antibodies. Co-immunization enhanced *Pneumococcus*-specific IgG and IgA relative to individual immunization with  $\gamma$ -PN (Figure 2A), demonstrating the ability of  $\gamma$ -FLU to enhance humoral responses.

We next investigated whether the combination vaccine modulated the CD4<sup>+</sup> cellular immune response. Following immunization, mice were depleted of CD4<sup>+</sup> cells prior to and during challenge with the colonization-induced pneumonia model. Co-immunized mice depleted of CD4<sup>+</sup> cells were no longer protected against pneumococcal disease (Figure 2B). In contrast, co-immunized mice given isotype control antibodies remained protected from pneumococcal challenge. Additionally, vaccination with  $\gamma$ -PN did not protect mice in the presence or absence of CD4<sup>+</sup> cells. These data indicate that vaccination with  $\gamma$ -PN +  $\gamma$ -FLU elicits enhanced protection against pneumonia that is dependent on CD4<sup>+</sup> cellular immune responses.

### Co-immunization enhances *Pneumococcus*-specific memory Th17 and Th1 cell responses

We have reported that IN vaccination with  $\gamma$ -PN promotes  $\gamma\delta$ T17 cells as opposed to Th17 cells [16]. To explore the CD4<sup>+</sup>-dependent immunity associated with enhanced protection induced by co-immunization, we investigated the nature of antigen-specific CD4<sup>+</sup> T-cell memory responses. We detected a significant increase in the total number of CD4<sup>+</sup> effector and Th17 cells in the lungs of  $\gamma$ -PN-vaccinated mice relative to unvaccinated mice following *ex vivo* stimulation with  $\gamma$ -PN (Figure 3A). This was associated with significant levels of IL-17 in the culture supernatants (Figure 3B). Co-immunization further enhanced lung CD4<sup>+</sup> effector, Th17 and Th1 cells, as well as IL-17 levels, relative to PBS and  $\gamma$ -PN vaccinated groups (Figures 3A and 3B). Moreover, we only detected Th17 cells in the spleens of co-immunized mice relative to PBS control mice and this also corresponded with significant levels of IL-17 in culture supernatants. These data indicate that co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU enhances CD4<sup>+</sup> effector cell generation, including *Pneumococcus*-specific Th1 and Th17 cells.



**Figure 1** Co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU enhances the protective efficacy of  $\gamma$ -PN

Mice were immunized IN with two doses of  $\gamma$ -PN,  $\gamma$ -FLU or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU ( $n = 10$ – $11$  mice/group). Two weeks after the second immunization, mice were challenged IN and monitored for survival after lethal D39 challenge (A). Alternatively, 2 weeks after the second immunization, mice were colonized IN with EF3030 and treated 4 days later with PBS IN (colonization-induced pneumonia model) and monitored for survival post-PBS treatment (B) or tested for bacterial counts in the lung and nasopharynx 7 days post-colonization (day 3 post-PBS treatment) (C). In addition, immunized mice were colonized IN with EF3030 (colonization model, no PBS treatment) and bacterial counts in the nasopharynx were assessed on day 7 post-colonization (D). Dotted line in (C) and (D) represents the minimum CFU detection limit. Statistical analysis was performed using one-way ANOVA (bacterial loads) and Fisher's exact test (survival rates); \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

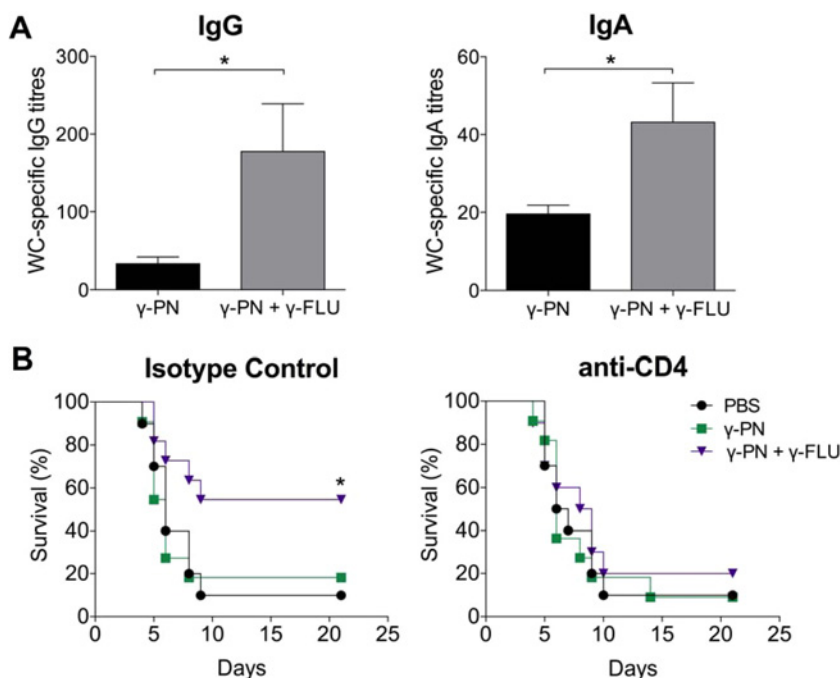
### Co-immunization promotes tissue-resident memory cell development

TRM cells are pivotal in providing immediate protection in defence against respiratory pathogens such as influenza [22–24]. We have shown that  $\gamma$ -PN vaccination does not elicit CD4<sup>+</sup> TRM cells [16]. Therefore, we were interested in determining whether the CD4<sup>+</sup>-dependent immunity observed with co-immunization is associated with the development of CD4<sup>+</sup> TRM cells. TRM cells were gated using the markers CD44 and CD103. These cells also displayed a CD11a<sup>hi</sup> surface phenotype (Figure 4A) [25,26]. Flow cytometry revealed a significant enhancement in CD4<sup>+</sup> and CD8<sup>+</sup> lung TRM cells from co-immunized mice (Figure 4A). Lung CD4<sup>+</sup> TRM cells from  $\gamma$ -FLU vaccinated mice were also increased relative to PBS controls. As expected [16], there were no significant alterations in CD4<sup>+</sup> and CD8<sup>+</sup> TRM cell populations from  $\gamma$ -PN vaccinated mice. Our data indicate that co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU, as well as immunization with  $\gamma$ -FLU alone, promotes TRM cell development. However, despite the apparent elevated levels of CD4<sup>+</sup> TRM in the co-

immunized group compared with mice immunized with  $\gamma$ -FLU alone, this difference did not reach significance.

### Co-immunization promotes Th17 and CD4<sup>+</sup>TRM IL-17<sup>+</sup> cell responses to live pneumococcal challenge

We next investigated the frequency of T-effector and TRM cell populations in the context of a live pneumococcal challenge following immunization with  $\gamma$ -PN and/or  $\gamma$ -FLU. In response to D39, co-immunized mice contained a higher frequency of CD4<sup>+</sup> T-effector cells relative to  $\gamma$ -FLU or  $\gamma$ -PN vaccinated mice. This elevation was associated with an increase in Th1 cells relative to  $\gamma$ -FLU or  $\gamma$ -PN vaccinated mice, and Th17 cells relative to  $\gamma$ -FLU vaccinated and PBS control mice (Figure 4B). Similarly, total, IL-17<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> TRM cells were significantly increased in co-immunized mice relative to all groups (Figure 4B). There was no impact on CD8<sup>+</sup> TRM cells for any group following D39 challenge (results not shown). These data suggest that co-immunization promotes lung Th17, CD4<sup>+</sup> TRM IL-17<sup>+</sup>



**Figure 2** The protective efficacy of the combination vaccine is dependent on CD4<sup>+</sup> responses

Mice were immunized IN with two doses of  $\gamma$ -PN or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU. Two weeks after the second immunization, sera were collected and analysed for pneumococcal-specific IgG or IgA titres by ELISA (A). Endpoint titres were presented as mean  $\pm$  S.E.M. ( $n = 8$  mice/group) and data were analysed using unpaired two-tailed Student's *t* test ( $*P < 0.05$ ). In addition, 2 weeks after the second immunization, mice were colonized with EF3030 and injected with anti-CD4 (150  $\mu$ g) or isotype control (rat IgG2b) at day -2, -1, 1 and 4 post-PBS treatment in the colonization-induced pneumonia model ( $n = 10$ –11 mice/group). Mice were monitored for survival post-PBS treatment for a period of 21 days (B). Survival rates were analysed using Fisher's exact test ( $*P < 0.05$ ).

and IFN- $\gamma$ <sup>+</sup> memory cell responses upon lethal pneumococcal challenge and implicate these subsets as the most likely cellular responses underlying the enhanced efficacy observed with the combination vaccine.

### Co-immunization does not compromise the protective efficacy of $\gamma$ -FLU

An important caveat when combining a whole bacterial ( $\gamma$ -PN) vaccine with an influenza ( $\gamma$ -FLU) vaccine is that dual vaccination does not compromise vaccine efficacy against each pathogen in isolation. Thus, to assess whether co-vaccination influenced anti-influenza immunity induced by  $\gamma$ -FLU, mice were challenged with A/PR8 and monitored for survival. Mice immunized with  $\gamma$ -FLU alone or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU were protected from lethal A/PR8 challenge (Figure 5). We conclude that co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU does not impair the protective efficacy of  $\gamma$ -FLU against influenza infection.

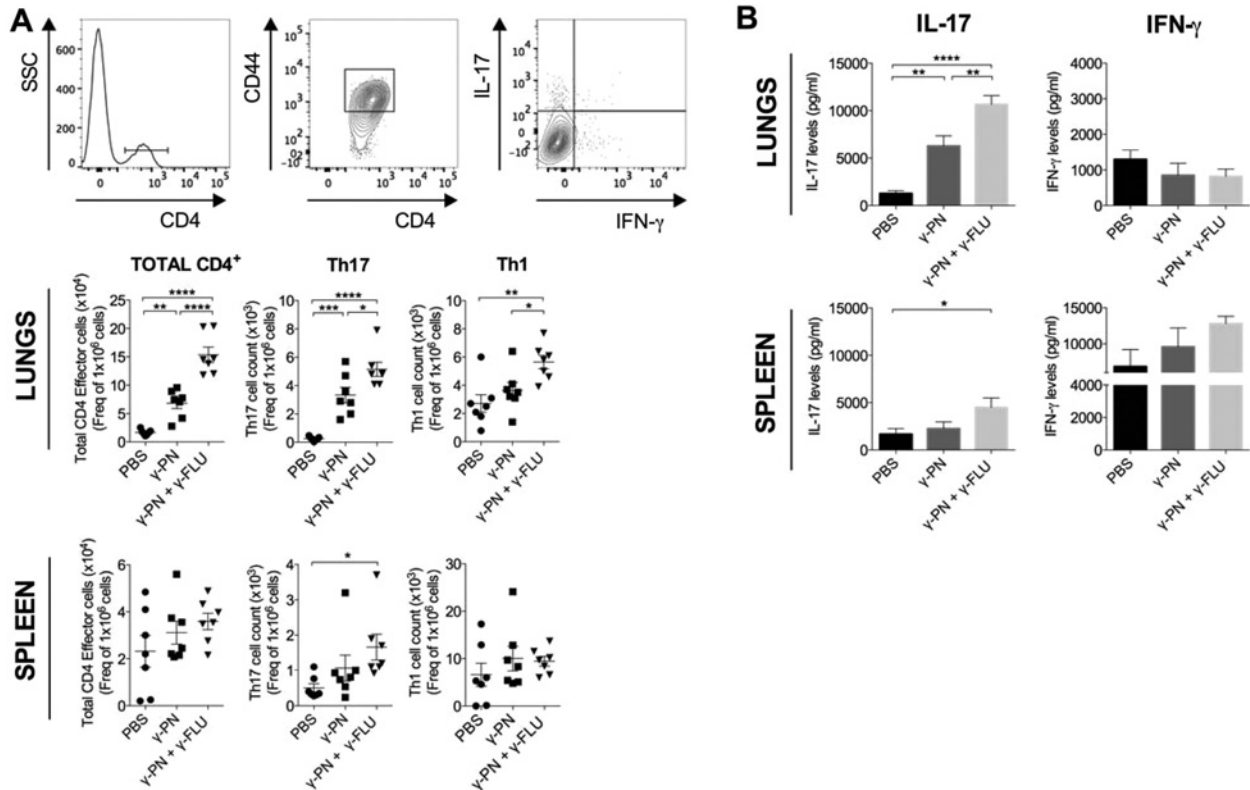
To further understand the impact of co-immunization on the nature of anti-FLU responses, we investigated influenza-specific CD4<sup>+</sup> T-cell memory responses. Lung suspensions from co-immunized mice re-stimulated *ex vivo* with  $\gamma$ -FLU contained greater frequencies of influenza-specific CD4<sup>+</sup> T-effector and Th17 cells relative to the PBS control. These populations were also enhanced in comparison with  $\gamma$ -FLU vaccinated mice (Figure 6A). Th17 cells were also significantly increased in the spleen

of co-immunized mice. In addition, Th1 cells in the lungs and spleens from co-immunized mice also appeared to be enhanced, but this did not reach significance. We also detected significant levels of IL-17 and IFN- $\gamma$  in supernatants from both lung and spleen cultures of co-immunized mice. IL-17 levels were further enhanced relative to  $\gamma$ -FLU vaccinated group in spleen cultures, but not the lung (Figure 6B). For  $\gamma$ -FLU vaccinated mice, we detected a significant increase in CD4<sup>+</sup> T-effector cells as well as IL-17 and IFN- $\gamma$  in lung cultures relative to PBS controls. Thus, our data confirm that co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU enhances lung influenza-specific CD4<sup>+</sup> effector cells and that this is associated with an unexpected increase in influenza-specific Th17 cells.

Lastly, we investigated the impact of the combination vaccine on the generation of antibody responses to influenza. Co-immunization did not alter influenza-specific antibody titres relative to individual vaccination with  $\gamma$ -FLU (Figure 6C). This further supports the notion that co-immunization does not compromise influenza-specific immune responses.

### Co-immunization elicits significant protection against co-infection

Co-infection with *S. pneumoniae* and influenza enhances bacterial growth in upper respiratory tract lavages, which is responsible for an exacerbation of infection [27]. Therefore, we



**Figure 3** Co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU enhances memory pneumococcal-specific Th17 and Th1 cells

Mice were immunized IN with two doses of  $\gamma$ -PN or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU ( $n = 7$ /group). Two weeks after the second immunization, the lung and spleen were harvested and single cell suspensions were re-stimulated in culture with the  $\gamma$ -PN vaccine ( $1 \times 10^6$  equivalent CFU/ml) or medium alone (negative control) for 72 h. (A) Frequency of live CD4<sup>+</sup> T-effector cell subsets in lung and spleen suspensions were determined by flow cytometry: total CD4<sup>+</sup> T-effector cells (CD4<sup>+</sup>CD44<sup>+</sup>), Th17 (CD4<sup>+</sup>CD44<sup>+</sup>IL-17<sup>+</sup>) and Th1 (CD4<sup>+</sup>CD44<sup>+</sup>IFN- $\gamma$ <sup>+</sup>). (B) Levels of cytokines (IL-17 and IFN- $\gamma$ ) in the supernatant of cultured lymphocytes from the spleens and lungs as determined by ELISA and presented as mean + S.E.M. Data were analysed using one-way ANOVA (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).

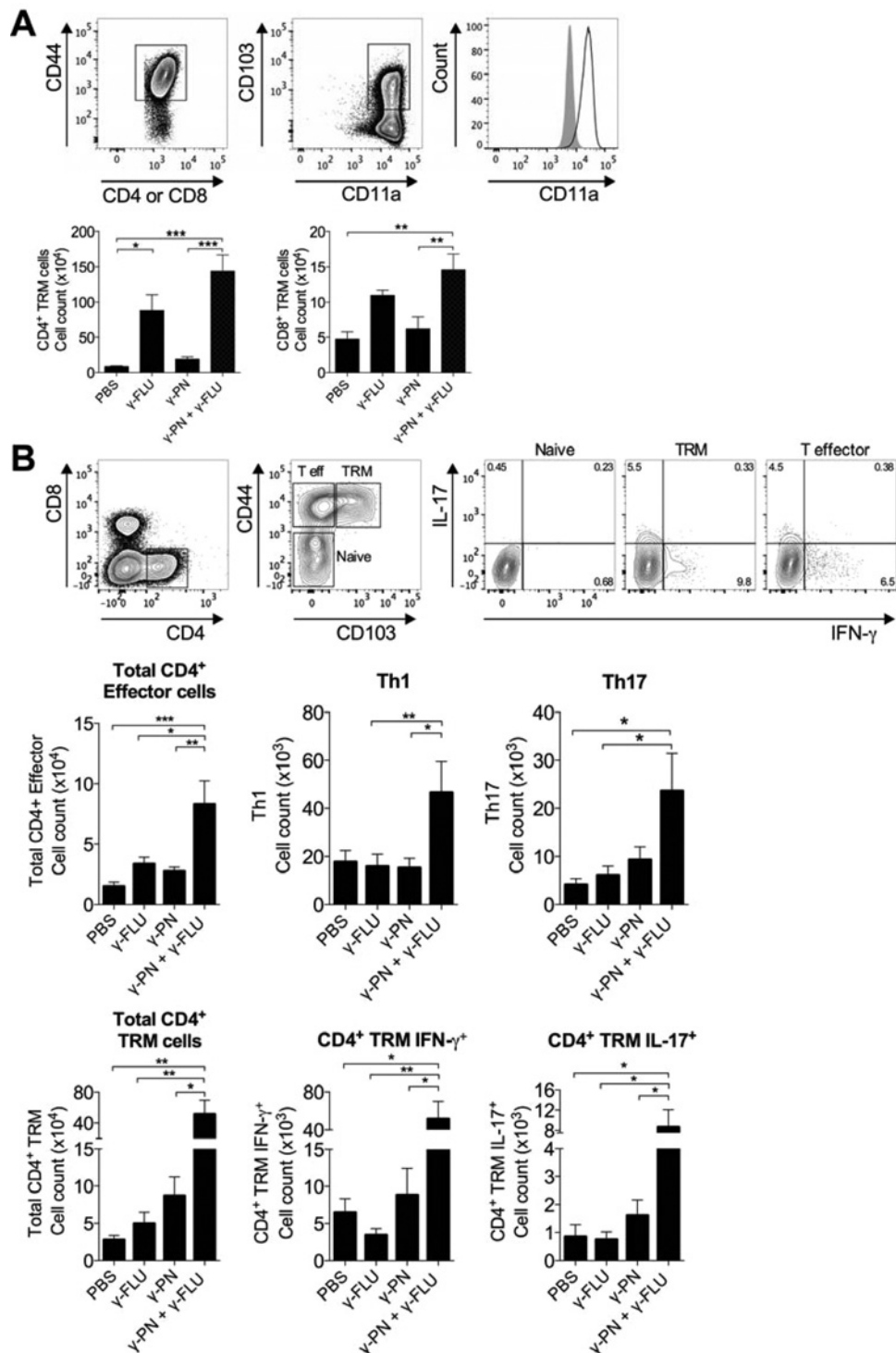
established a co-infection model to determine the efficacy of the  $\gamma$ -PN +  $\gamma$ -FLU combination vaccine against co-infection. Three groups of mice were colonized IN with *S. pneumoniae* EF3030 and 4 days later the mice were either anaesthetized and challenged IN with a sublethal dose of A/PR8 (co-infection; PN + FLU) or treated with PBS [to establish pneumonia (PN)]. Mice administered PBS IN without anaesthetic (NO AN) served as a colonization only control group. Seven days later, CFU recovery was determined in the nasopharynx and lung. As expected, co-infected mice (PN + FLU) showed greatly enhanced bacterial counts in both the nasopharynx and lung relative to the PN or the colonization only group (NO AN) (Figure 7A). 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  $\gamma$ -PN +  $\gamma$ -FLU vaccine against co-infection was next investigated. Mice co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU were the only group that displayed significant protection relative to unvaccinated (PBS) control mice in the co-infection model (PN + FLU). These data confirm that co-immunization enhances the vaccine efficacy of  $\gamma$ -PN against pneumococcal pneumonia,

as well as conferring significant protection against co-infection with both *S. pneumoniae* and influenza.

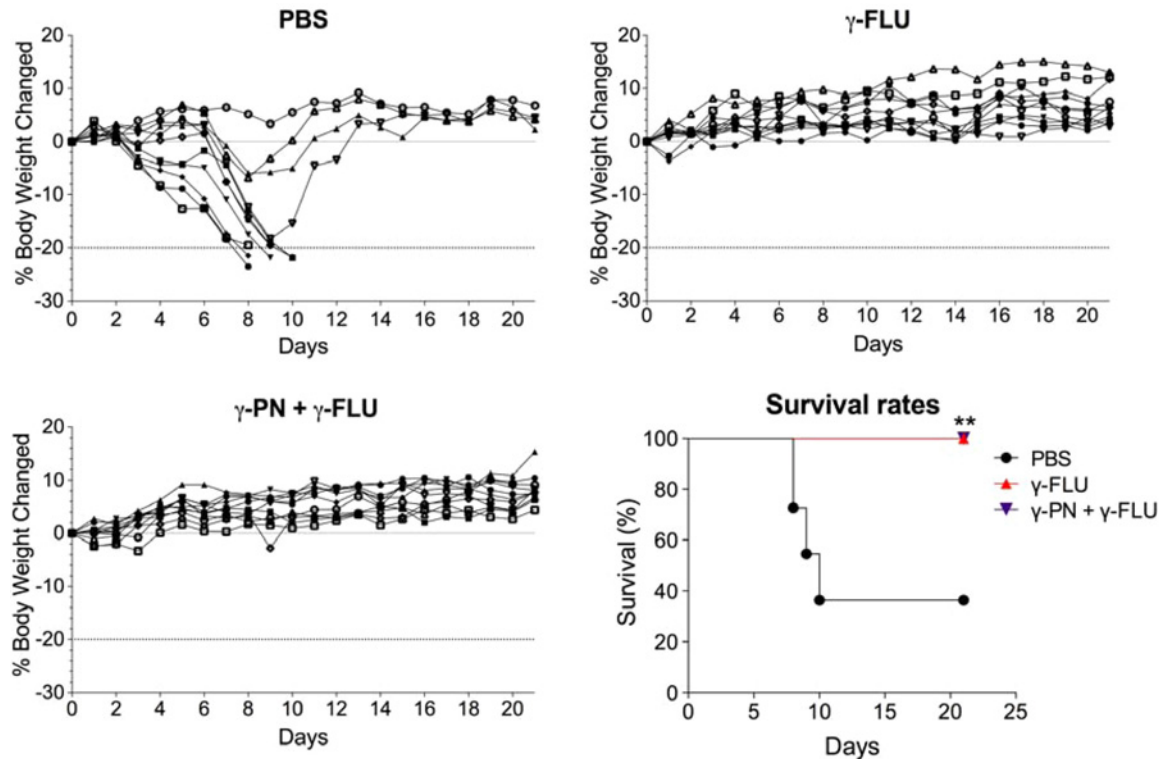
## DISCUSSION

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 [7–9], challenges still remain with current vaccine strategies as bacteria or viral strains can escape vaccine-induced immunity. In the present study, we demonstrate that intranasal co-vaccination with  $\gamma$ -PN +  $\gamma$ -FLU does not compromise protective efficacy of  $\gamma$ -PN or  $\gamma$ -FLU vaccination against each pathogen. Surprisingly, our data indicate that the combination of the two vaccines significantly enhances protective efficacy of  $\gamma$ -PN against pneumococcal pneumonia and co-infection. 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.



**Figure 4** Co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU induces Th17 and CD4<sup>+</sup> TRM cells

Mice were immunized IN with two doses of  $\gamma$ -PN,  $\gamma$ -FLU or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU ( $n=6$ /group). Two weeks following the second immunization, (A) the lungs were harvested and analysed by flow cytometry for CD4<sup>+</sup> and CD8<sup>+</sup> TRM cells (CD44<sup>+</sup>CD103<sup>+</sup>CD11a<sup>hi</sup>). (B) Immunized mice were also challenged IN with D39, and lungs were harvested 48 h post-challenge and analysed by flow cytometry for total CD4<sup>+</sup> T-effector cells (CD4<sup>+</sup>CD44<sup>+</sup>CD103<sup>-</sup>), Th17 (CD4<sup>+</sup>CD44<sup>+</sup>CD103<sup>-</sup>IL-17<sup>+</sup>), Th1 (CD4<sup>+</sup>CD44<sup>+</sup>CD103<sup>-</sup>IFN- $\gamma$ <sup>+</sup>); and total CD4<sup>+</sup> TRM cells (CD4<sup>+</sup>CD44<sup>+</sup>CD103<sup>+</sup>), CD4<sup>+</sup> TRM IL-17<sup>+</sup> cells (CD4<sup>+</sup>CD44<sup>+</sup>CD103<sup>+</sup>IL-17<sup>+</sup>) and CD4<sup>+</sup> TRM IFN- $\gamma$ <sup>+</sup> cells (CD4<sup>+</sup>CD44<sup>+</sup>CD103<sup>+</sup>IFN- $\gamma$ <sup>+</sup>). Data were presented as mean  $\pm$  S.E.M. and analysed using one-way ANOVA (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



**Figure 5** Co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU does not compromise vaccine-induced anti-influenza immunity

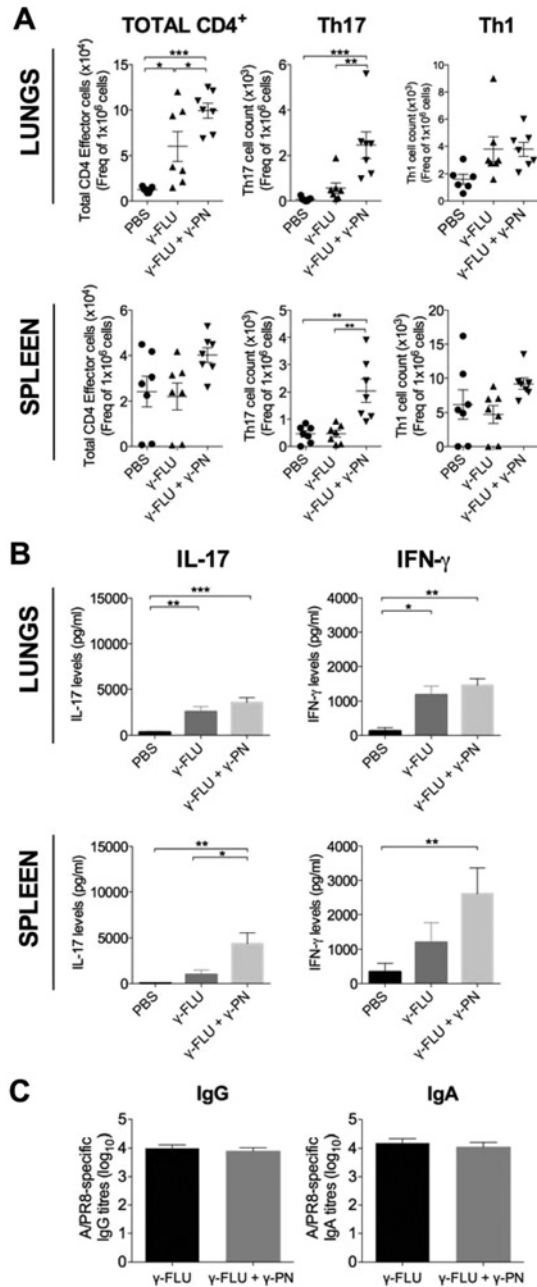
Mice were immunized IN with two doses of  $\gamma$ -FLU or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU ( $n=10$ /group). Two weeks after the second immunization, mice were challenged IN under anaesthesia with A/PR8 and mice were monitored for a period of 21 days for weight loss. Animals were culled if they lost 20% of their starting body weight (shown as dotted line) and data were presented as percentage survival and analysed using Fisher's exact test (\*\* $P < 0.01$ ).

Our pneumonia and co-infection models rely on the ability of pneumococci to colonize the nasopharynx and migrate into the lung to establish focal pneumonia upon intranasal delivery of PBS or influenza. Reduced nasopharyngeal bacterial counts detected in co-immunized mice confirm that the combination vaccine mediates protection at the stage of colonization. Immunity against nasopharyngeal carriage is mainly dependent on Th17 cells, with a limited role for B-cell responses [28–30]. Previous studies have reported the efficacy of a cholera-toxin adjuvanted pneumococcal vaccine to induce Th17-dependent protection against colonization [29,31]. We previously reported that serotype-independent protection elicited by the un-adjuvanted  $\gamma$ -PN vaccine was mediated by B-cells and innate IL-17 responses, but not Th17 cells [16]. In the present study, we demonstrate that co-immunization elevated *Pneumococcus*-specific antibody responses, which support previous work regarding the ability of  $\gamma$ -FLU to enhance humoral responses to a co-administered antigen [17]. In addition, our data indicate that CD4<sup>+</sup> T-cells play a key role in the observed enhancement of vaccine efficacy, as humoral immune responses alone were not sufficient to mediate protection in CD4<sup>+</sup> depleted mice. In particular, we observed a significant induction of antigen-specific Th17 cells in co-immunized mice relative to individual vaccination with  $\gamma$ -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 co-immunized mice. Nevertheless, augmented antibody responses may still be playing a minor role. We also observed detectable Th17 cells in cultures from  $\gamma$ -PN vaccinated mice, but not in response to live challenge, which is consistent with earlier reports [16]. This may explain lack of protection in these mice against colonization-induced pneumonia.

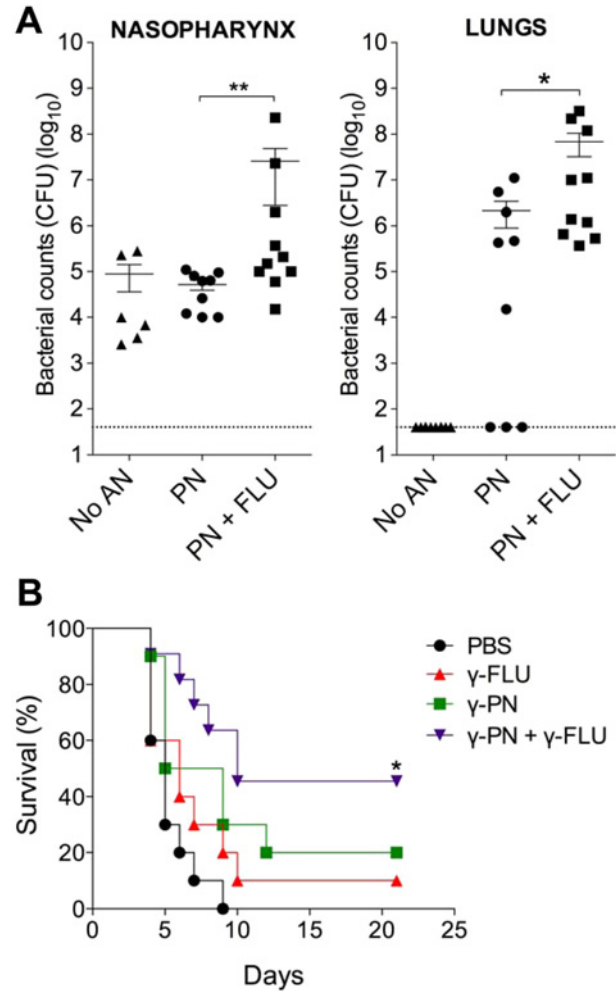
TRM cells are a unique memory subset that localize and remain in specific tissues, such as the lung, following primary infection [32]. To our knowledge, there have been no reports regarding the development of TRM cells during pneumococcal infection, nor has this been anticipated as a part of pneumococcal vaccine design. We previously showed that  $\gamma$ -PN vaccination does not promote TRM development [16]. In the present study, we detected CD4<sup>+</sup> and CD8<sup>+</sup> TRM cells in mice co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU. In particular, there was a profound increase in CD4<sup>+</sup> IL-17<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> TRM cells in response to live pneumococcal challenge. Although antigen-specificity was not defined, these memory cells may be *Pneumococcus*-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 [24]. Therefore, TRM cell





**Figure 6** Co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU enhances memory influenza-specific Th17 cells

Mice were immunized IN with two doses of  $\gamma$ -FLU or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU ( $n=7$ /group). Two weeks after the second immunization, the lungs and spleens were harvested and single cell suspensions were re-stimulated in culture with the  $\gamma$ -FLU vaccine ( $2 \times 10^7$  equivalent TCID<sub>50</sub>/ml) or medium alone (negative control) for 72 h. (A) Frequency of live CD4<sup>+</sup> T-effector cell subsets in lung and spleen suspensions were determined by flow cytometry (gated as in Figure 3A). (B) Levels of cytokines (IL-17 and IFN- $\gamma$ ) in the supernatant of cultured lymphocytes from the spleens and lungs as determined by ELISA and presented as mean  $\pm$  S.E.M. (C) Serum influenza-specific IgG and IgA endpoint titres 2 weeks after the second immunization as determined by ELISA ( $n=8$ /group) and data presented as mean  $\pm$  S.E.M. Data were analysed using one-way ANOVA (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



**Figure 7** Co-immunization with  $\gamma$ -PN +  $\gamma$ -FLU provides significant protection against co-infection

(A) Mice were colonized with EF3030 and 4 days later treated under anaesthesia with either PBS (PN; colonization-induced pneumonia model) or a sublethal dose of A/PR8 (PN + FLU; co-infection model). Another group of mice were administered PBS without anaesthetic (NO AN; colonization model). Bacterial counts in the nasopharynx and lungs were determined 7 days later. Data were presented as mean  $\pm$  S.E.M. and analysed using one-way ANOVA (\* $P < 0.05$ , \*\* $P < 0.01$ ). (B) Mice were immunized IN with two doses of  $\gamma$ -PN,  $\gamma$ -FLU or co-immunized with  $\gamma$ -PN +  $\gamma$ -FLU ( $n=10-11$  mice/group). Two weeks after the second immunization, mice were infected IN with EF3030 and then administered A/PR8 IN 4 days later (co-infection model). Mice were monitored for 21 days and data were presented as percentage survival and analysed using Fisher's exact test (\* $P < 0.05$ ).

induction by the combination vaccine, in addition to high levels of antibodies, Th17 and Th1 responses, could facilitate optimal clearance of pneumococci in comparison with individual vaccination with  $\gamma$ -PN, likely through IL-17/IFN- $\gamma$  driven recruitment of phagocytic cells or antibody-mediated opsonophagocytosis.

We have recently reported that  $\gamma$ -FLU enhances the immunogenicity of a co-administered viral vaccine most likely via IFN-I [17]. In the context of co-infection, high IFN-I levels induced by influenza have been reported to inhibit both Th17 [33] and

$\gamma\delta$ T-cell responses [34]. Although  $\gamma$ -FLU is a strong inducer of IFN-I [35], we observed accelerated Th17 responses in co-immunized mice. This suggests that an IFN-I-related inhibitory effect on Th17 responses does not occur in the context of our co-vaccination strategy. The molecular mechanism by which  $\gamma$ -FLU governs the induction of Th17 responses when in combination with  $\gamma$ -PN was not evaluated in the present study. However, previous studies suggest a possible involvement of TLR4. Immunization with an inactivated influenza strain H5N1 has been reported to induce the generation of reactive oxygen species that triggers the production of oxidized phospholipids (OPLs) in lung airways. OPLs were shown to induce the production of IL-6 through TLR4 signalling in alveolar macrophages [36]. In addition, the involvement of TLR4 signalling to promote the generation of IL-17 differentiating cells has been reported *in vitro* [37] and *in vivo* [38,39]. Thus, vaccination with  $\gamma$ -PN +  $\gamma$ -FLU could promote signalling through TLR4 to induce IL-6, potentially driving Th17 development, but this remains to be tested.

Interestingly, the combination vaccine also induced high levels of influenza-specific Th17 cells. The role of Th17 immunity during influenza infection is controversial and it remains unclear whether it is beneficial [40] or detrimental to the host [39,41]. Although we have not investigated the possible source of IL-17 in  $\gamma$ -FLU vaccinated mice, we demonstrate that Th17 cell induction in co-immunized mice did not compromise the protective efficacy of  $\gamma$ -FLU.

In conclusion, the present study provides the first evidence illustrating the ability 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. We also demonstrate that the combined vaccine can induce effective protection against each pathogen, and against co-infection. Considering the effectiveness reported with  $\gamma$ -PN and  $\gamma$ -FLU vaccines individually, this vaccination strategy heralds the possibility a simplified ‘universal’ immunization strategy for protecting against diverse serotypes and strains of both *S. pneumoniae* and influenza.

## CLINICAL PERSPECTIVES

- Influenza-induced susceptibility to secondary bacterial pneumonia is responsible for the excess mortality that occurs during influenza pandemics. Current strategies to reduce the risk of co-infection are focused on developing single vaccines against *S. pneumoniae* and influenza. However, challenges still remain with the current individual vaccines that allow bacterial or viral strains to escape vaccine-induced immunity and cause significant disease. In the present study, we investigated the efficacy of a mucosal combination vaccine comprising  $\gamma$ -irradiated pneumococcal whole cells ( $\gamma$ -PN) and  $\gamma$ -irradiated influenza virions ( $\gamma$ -FLU).
- The results of the present study show that intranasal vaccination with the combination vaccine provided significant protection against individual infection and co-infection with both *S. pneumoniae* and influenza. The present study also reports the

ability of the  $\gamma$ -irradiated influenza vaccine to enhance the immunogenicity of the pneumococcal vaccine by favouring the induction of Th17 and CD4<sup>+</sup> TRM cells.

- This finding heralds the possibility of a simplified ‘universal’ immunization strategy for protecting against diverse serotypes and strains of both *S. pneumoniae* and influenza.

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## DECLARATIONS OF INTEREST

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

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## AUTHOR CONTRIBUTION

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

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