



Effect of a pneumococcal whole cell vaccine on influenza A-induced pneumococcal otitis media in infant mice

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ABSTRACT

The pneumococcus remains a common cause of otitis media (OM) despite the widespread introduction of pneumococcal conjugate vaccines. In mice, a pneumococcal whole cell vaccine (WCV) induces serotype-independent protection against pneumococcal colonisation and invasive disease via T_H17- and antibody-mediated immunity, respectively. We investigated the effect of WCV on influenza A-induced pneumococcal OM in an infant mouse model.

C57BL/6 mice were immunised subcutaneously with a single dose of WCV or adjuvant at 6 days of age, infected with pneumococci (EF3030 [serotype 19F] or PMP1106 [16F]) at 12 days of age, and given influenza A virus (A/Udorn/72/307 [H3N2], IAV) at 18 days of age to induce pneumococcal OM. Pneumococcal density in middle ear and nasopharyngeal tissues was determined 6 and 12 days post-virus. Experiments were repeated in antibody (B6.μMT^{-/-})- and CD4⁺ T-cell-deficient mice to investigate the immune responses involved.

A single dose of WCV did not prevent the development of pneumococcal OM, nor accelerate pneumococcal clearance compared with mice receiving adjuvant alone. However, WCV reduced the density of EF3030 in the middle ear at 6 days post-viral infection ($p = 0.022$), and the density of both isolates in the nasopharynx at 12 days post-viral infection (EF3030, $p = 0.035$; PMP1106, $p = 0.011$), compared with adjuvant alone. The reduction in density in the middle ear required antibodies and CD4⁺ T cells: WCV did not reduce EF3030 middle ear density in B6.μMT^{-/-} mice ($p = 0.35$) nor in wild-type mice given anti-CD4 monoclonal antibody before and after IAV inoculation ($p = 0.91$); and WCV-immunised CD4⁺ T cell-deficient GK1.5 mice had higher levels of EF3030 in the middle ear than their adjuvant-immunised counterparts ($p = 0.044$).

A single subcutaneous dose of WCV reduced pneumococcal density in the middle ears of co-infected mice in one of two strains tested, but did not prevent OM from occurring in this animal model.

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1. Introduction

Otitis media (infection of the middle ear; OM) is a common paediatric condition, and *Streptococcus pneumoniae* (pneumococcus) is

a leading cause [1,2]. OM typically arises when bacteria disseminate from the nasopharynx, where the pneumococcus resides in up to 90% of healthy children under the age of five [3], to the middle ear cavity via the Eustachian tube. Young children suffer the highest rates of OM. Pneumococcal conjugate vaccines (PCVs) induce immunity against the pneumococcal polysaccharide capsule and are highly effective against invasive pneumococcal disease [4]. However, their impact on OM has been more variable. Clinical trials evaluating the effect of the 7- and 13-valent PCVs demonstrated a 25–

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56% reduction in acute otitis media (AOM) caused by any pneumococcus, and a ~60% reduction in AOM caused by PCV-type pneumococci [5–7]. Importantly, PCVs only protect against a small subset of pneumococcal serotypes and their use has led to increases in disease caused by non-vaccine serotypes [8]. Alternative vaccines which induce protection against non-capsular pneumococcal antigens may provide enhanced protection against OM compared to PCVs.

The pneumococcal whole cell vaccine (WCV) consists of a killed, unencapsulated pneumococcal strain delivered with adjuvant [9]. WCV was developed to induce broad protection against all pneumococci at low cost. Preclinical studies have demonstrated WCV to be protective against both invasive pneumococcal disease and colonisation in mice [9,10] and have provided sufficient evidence to allow the progression of WCV into clinical trials [11]. Only one preclinical study has examined the ability of WCV to prevent pneumococcal OM. Malley et al. tested the effect of two or three doses of WCV, consisting of ethanol-killed whole cell antigen (WCA) combined with various adjuvants, given intranasally (i.n.) to adult mice before i.n. challenge with pneumococci [12]. Pneumococcal density was enumerated in nasopharyngeal and middle ear washes one week after challenge and compared to that in mice which received adjuvant alone. Each schedule of WCV (with cholera toxin adjuvant) significantly reduced the number of mice colonised with a serotype 6B isolate, and also reduced the density of nasopharyngeal colonisation [12]. Further, WCV significantly protected mice from middle ear infection with a serotype 23F isolate [12]. Despite these promising results, it is unclear whether WCV prevented the migration of pneumococci into the middle ear, or if WCV-mediated responses accelerated the clearance of pneumococci following the development of infection, as was observed for WCV-mediated protection against colonisation [13]. Moreover, the immune mechanisms involved in protection of WCV against OM have not been investigated.

Co-infection with a respiratory virus is associated with OM in young children [14,15]. We have developed an infant mouse model of pneumococcal OM [16] in which pneumococcal colonisation is induced experimentally by delivering a small dose of the bacterium to the nares of an unanaesthetised animal. Pneumococci remain localised in the upper respiratory tract for approximately five weeks [17], mimicking a carriage episode similar to that observed in young children [18,19]. Subsequent i.n. co-infection of colonised mice with influenza A virus (IAV) causes pneumococci to multiply in the nasopharynx and disseminate to the middle ear cavity, resulting in OM and hearing loss [16]. In this study, we adapted this model to investigate the effect of WCV on IAV-induced pneumococcal OM in infant mice.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Animal Ethics Committee of the University of Melbourne (1413144) and were conducted in accordance with the Prevention of Cruelty to Animals Act (1986) and the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes (2013). C57BL/6 (WT), B6.μMT^{-/-} and GK1.5 mice were bred and housed at the Biological Resources Facility (BRF), Department of Microbiology and Immunology at The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia. Litters of mice were housed in ventilated cages with unlimited access to sterile food and water.

2.2. Bacterial and viral strains

Pneumococcal isolates EF3030 (serotype 19F) and PMP1106 (serotype 16F) were passaged in mice and recovered from the

nasopharynx before experimental use. IAV strain A/Udorn/72/307 (H3N2, A/Ud/72) was used in all experiments. Bacterial and viral infectious stocks were prepared as described previously [20].

2.3. Vaccine

WCV was prepared as described previously [21]. Whole cell antigen (WCA) consisted of pneumococcal strain RM200 (a derivative of the unencapsulated pneumococcal strain RX1E containing a non-toxic pneumolysin and deletion of the autolysin gene [22]) killed by treatment with beta-propiolactone. Each 100 μl WCV dose contained 100 μg of WCA and 2.5 μg/μl aluminium phosphate (Alhydrogel [Brenntag]) in sterile saline. In all vaccination experiments the effects of WCV were compared to a group of mice receiving the same dose of Alhydrogel in 100 μl of saline (adjuvant).

2.4. Otitis media model

Litters of 12-day-old mice were infected i.n. with $\sim 2 \times 10^3$ CFU of pneumococci in 3 μl of PBS followed by i.n. infection with $\sim 10^{2.5}$ PFU of IAV in 3 μl of PBS 6 days later. In vaccination experiments, mice were first immunised with WCV or adjuvant via subcutaneous injection to the back of the neck at 6 days of age, followed by co-infection as described above. Mice were euthanised by CO₂ inhalation at predetermined time points post-infection. Nasopharyngeal and middle ear tissue were collected for enumeration of pneumococci by culture on gentamicin horse blood agar as described previously [20]. IAV was quantified by reverse transcriptase qPCR (RTqPCR, see below). Blood samples and spleens were harvested and analysed for immune responses as described below. To obtain plasma, whole blood was collected in heparinised tubes and samples were incubated overnight at 4 °C. Red blood cells were removed by centrifugation (2400 xg for 5 min followed by a further 11,300 xg at 4 °C for 5 min). Samples were stored at -80 °C until tested. To obtain single cell suspensions of splenocytes, spleens were mechanically disassociated by passing the tissue through a 70 μm cell strainer (Falcon). Splenocytes were collected by centrifugation (350 xg at 4 °C for 5 min) and resuspended in 2 ml of TAC buffer (containing a final concentration of 17 mM Tris and 140 mM ammonium chloride) at room temperature for 10 min with infrequent inversion to lyse red blood cells. RPMI 1640 medium (Sigma-Aldrich) was added to each sample to stop the reaction and splenocytes were washed and collected by centrifugation as above and resuspended in 1 ml Dulbecco's Modified Essential Medium with Ham's F12 medium (DMEM/F12 [Gibco]).

To investigate the immune mechanisms involved in mediating WCV effects on pneumococcal OM, mice deficient in antibodies (B6.μMT^{-/-} mice [23]), CD4⁺ T cells (GK1.5 mice [24]), or CD4⁺ T cells at the time of OM (WT mice treated with GK1.5 monoclonal antibody [mAb]) were co-infected as described above with additional modifications. As previous studies have determined that antibodies are required for the development of OM in co-infected B6.μMT^{-/-} mice [25], such mice were administered serum obtained from naive WT adult mice via intraperitoneal (i.p.) injection one day before, and each day following, IAV infection (aged 17, 19–23 days-old respectively). To deplete CD4⁺ T cells at the time of OM induction, GK1.5 mAb (Walter and Eliza Hall Institute [WEHI]) was administered via i.p. injection one day before, and two days after, IAV infection (aged 17 and 20 days-old respectively). Each dose consisted of 125 μg of antibody per 10 g of body weight in a total volume of 50 μl. The mAb rat isotype IgG2b antibody (WEHI) was administered to a control group at the same concentration to control for non-specific effects of antibody injection.

2.5. IAV RTqPCR

To quantify viral loads in mouse samples, tissues were processed as described previously [20] and viral RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was used as a template for one-step cDNA synthesis and RTqPCR using previously published primer and probe sequences amplifying the matrix gene of IAV [26]. RTqPCR reactions were performed in duplicate using the Stratagene MxPro 3005. Each 20 μ l reaction contained 4 μ l of extracted RNA, 10 μ l of SensiFAST probe No-ROX one-step master mix, 0.2 μ l of reverse transcriptase, 0.2 U/ μ l RiboSafe RNase inhibitor (all Biotline), 400 nM of each primer (Sigma-Aldrich) and 100 nM probe (Sigma-Aldrich). RTqPCR conditions were as follows: 1 cycle of 10 min at 45 °C and 1 cycle of 2 min at 95 °C followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. To quantify the number of matrix gene copies in each sample, plasmid DNA extracted from an *E. coli* strain transformed with the 4007 bp pHW plasmid containing the matrix protein from Ud/A/72 (XLI-B pHWUd/A/72matrix) was used to generate a standard curve. IAV loads are expressed as genome copies/ml based on 1 mg of plasmid DNA being equivalent to 227.7 IAV virions (assuming one copy of the matrix gene was present in each *E. coli* plasmid).

2.6. Histology

To analyse inflammation in the middle ear cavity, haematoxylin and eosin-staining was performed [58]. Mice were euthanised and were perfused intracardially with 0.4 g/L paraformaldehyde. The middle ears were dissected and fixed for at least 1 h in 0.4 g/L paraformaldehyde (Sigma-Aldrich). Tissues were washed three times in Tris Buffered Saline (TBS), containing a final concentration of 0.05 M Tris-HCl [Sigma-Aldrich] and 9 g/L sodium chloride [Sigma-Aldrich], pH 7.6. Tissues were then incubated for seven days in decalcification buffer (100 g/L EDTA [Sigma-Aldrich] in PBS, pH 7.2–7.4) before infiltration with TBS containing 3 g/L sucrose. Decalcified tissues were embedded in a 1:1 ratio of TBS + 3 g/L sucrose and Tissue-Tek[®] Optimal Cutting Temperature compound (OCT [Sakura Finetek Inc]) overnight at 4 °C before being transferred to pure OCT and further incubated overnight at 4 °C. OCT was solidified using isopentane in a container with liquid nitrogen. Sections of 2 μ m tissue were cut using the HM525 NX Cryostat (ThermoFisher Scientific), fixed onto glass slides and stained with haematoxylin (Sigma-Aldrich) for 2 min to identify cell nuclei, washed in distilled water, and then Scott's tap water (Sigma-Aldrich) was applied for 1 min. Sections were washed in distilled water and then counterstained with 10 g/L eosin (Sigma-Aldrich) and washed again with distilled water. Slides were dehydrated with absolute ethanol (3 \times 1 min), cleared with xylene (3 \times 1 min [Sigma-Aldrich]) and mounted with a coverslip. Slides were visualised using the Leica DM 1000 light microscope (Leica Microsystems) and scored blindly for inflammatory infiltrate in the middle ear cavity by an independent pathologist as described previously [16].

2.7. ELISA and ex vivo stimulation of whole blood

WCA-specific IgG was detected in plasma or in whole blood following ex vivo stimulation with vaccine antigen (WCA), or PBS as a control, using the method described by Lu et al. [9] with some modifications. 96-well ELISA plates (Maxisorp immuno plate [Nunc]) were coated overnight with 100 μ g of WCA and blocked with 10 g/L BSA in PBST (PBS + 0.5 ml/L Tween20 [Sigma-Aldrich]). Samples were diluted in PBST and added to duplicate wells for 2 h at 37 °C. Anti-mouse IgG biotin-conjugated antibody (Sigma-Aldrich) was added to each well at a dilution of 1 in

5000, and plates were incubated for 1 h at 37 °C. To detect antigen-IgG complexes, wells were washed and horseradish peroxidase (HRP)-labelled Streptavidin (Streptavidin-HRP [KPL]) was added to each well at a dilution of 1 in 5000 and incubated for 1 h at room temperature. Bound streptavidin-HRP was visualised using TMB substrate (KPL) and the optical density at 450 nm was read using a CLARIOstar plate reader (BMG Labtech) or an ELx808 Absorbance reader (BioTek). A standard curve was run on each plate using a 1 in 2 dilution series of positive control sera collected from adult mice which had received two subcutaneous doses of WCV at two-week intervals, two weeks after the second dose. A 1 in 1000 dilution of the control sera was assigned a value of 1000 arbitrary units (AU), and anti-WCA IgG responses in samples were interpolated from the standard curve.

IL-17A was measured in supernatants from splenocytes stimulated as described previously [13] using the Quantikine Mouse IL-17 ELISA kit (R&D Systems Inc.) according to the manufacturer's instructions.

2.8. Flow cytometry

The depletion of CD4⁺ T cells in mice treated with GK1.5 or rat IgG2b mAb antibody was confirmed by flow cytometry. Splenocytes were prepared as described above and labelled with fluorochrome conjugated antibodies specific for cell surface markers CD4 (clone RM4.4 [BD Horizon]), CD3 (clone 17A2 [BD Horizon]) and Thy1.2 (clone 53-2.1 [eBioscience]). The splenocyte samples were analysed using an LSRII flow cytometer (BD Bioscience) according to the manufacturer's instructions. Dead cells were identified and excluded in each sample by the addition of 1 μ g/10⁶ cells of propidium iodide (PI [Sigma-Aldrich]) immediately before analysis. For all samples, 1 \times 10⁵ events were collected and recorded.

2.9. Statistics

Data were analysed using GraphPad Prism version 6.0 h for MacIntosh and Stata version 15.1. Experimental groups were compared using the Mann Whitney *U* Test (Prism). A two-tailed Fisher's exact test was used to compare differences in the number of mice which developed unilateral OM, bilateral OM or no OM (Stata). The statistical test used for each analysis is indicated in the accompanying text and figure legends. *P* values less than 0.05 were considered statistically significant.

3. Results

To investigate the effect of WCV immunisation on OM, we first adapted our published model [16]. Mice were colonised with pneumococci at 12 days of age and then infected with IAV at 18 days of age (Fig. 1A). Pneumococcal isolates were chosen to represent serotypes that are commonly carried (19F) or that have been increasingly detected (16F) in carriage and OM in high-risk populations following PCV introduction [27,28]. Both pneumococcal isolates tested (EF3030 and PMP1106) were able to disseminate to the middle ears post-IAV administration (Fig. 1B and C). Co-infection induced pneumococcal OM in 16 of 17 mice colonised with EF3030 (94.1%) and 17 of 18 mice colonised with PMP1106 (94.4%) at six days post-IAV. The majority of mice had cleared the bacteria from the middle ears by 14 days post-IAV infection (Fig. 1B and C). Six days after IAV-infection, most co-infected mice had bilateral pneumococcal OM, regardless of the pneumococcal isolate used (Fig. 1D and E). During clearance, the proportion of mice with bilateral OM decreased, with a resultant increase in mice with unilateral and then no OM (Fig. 1D and E), indicating that

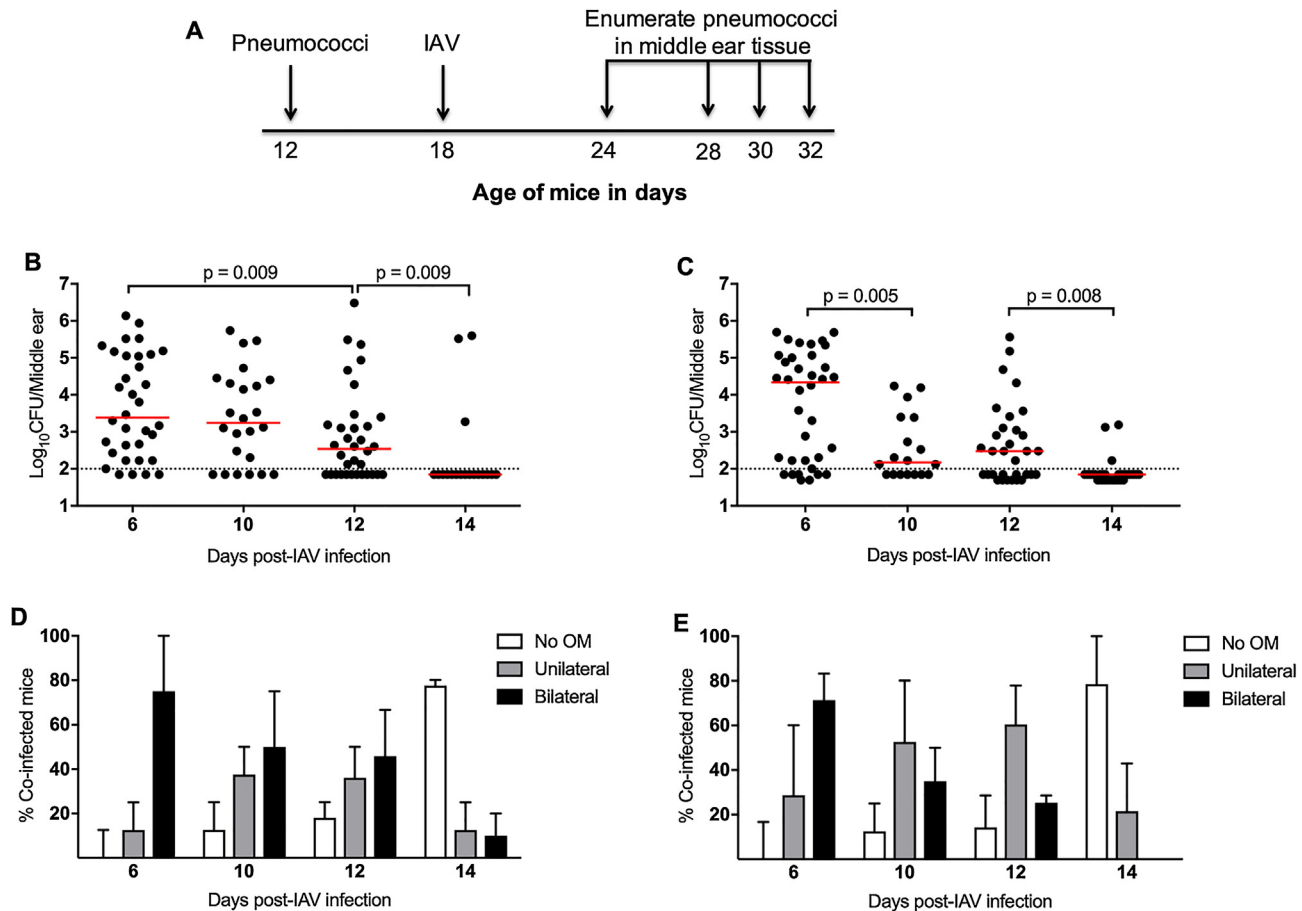


Fig. 1. Development and clearance of OM in mice co-infected with pneumococci and IAV. Mice were co-infected with pneumococci and IAV (A). At various time points post-IAV infection, pneumococcal density in the middle ear tissues was determined in mice colonised with EF3030 (B) or PMP1106 (C). Counts from each middle ear are represented as individual data points with red lines depicting the median. The dotted line represents the limit of detection (100 CFU). The proportion of co-infected mice with EF3030 (D) or PMP1106 (E) detected in neither (no OM, white bars), one (unilateral OM, grey bars) or both (bilateral OM, black bars) middle ears over time was determined. The error bars show the 95% confidence interval of the median. All graphs show data pooled from at least two independent experiments per time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

co-infected mice clear pneumococci from each ear independently. IAV was only detected in the middle ears of 2 of 32 (EF3030) and 2 of 26 (PMP1106) co-infected mice at 6 days post-IAV and was not detected in the middle ears after this time point.

To evaluate the effect of WCV on IAV-induced pneumococcal OM at the peak of disease, infant mice were vaccinated with a single subcutaneous dose of WCV (or adjuvant) to the back of the neck at 6 days of age, and then co-infected as shown in Fig. 1A. Pneumococcal and IAV density was measured in nasopharyngeal and middle ear tissue 6 days after infection with IAV. WCV significantly reduced the density of EF3030 in the middle ears of co-infected mice (Fig. 2A, $p = 0.022$), despite the mice having comparable levels of IAV at this site (Fig. 2C, $p = 0.93$). In 4 of 21 (19%) of WCV-immunised mice, EF3030 was not detected in either ear, whereas all 18 mice treated with adjuvant had detectable EF3030 in at least one ear (Table 1). Of mice with detectable EF3030 in any ear, bilateral OM was observed in 11 of 21 (52%) of mice administered WCV compared to 13 of 18 (72%) of the adjuvant control group (Table 1). No significant differences were observed in the median density of EF3030 in the nasopharynx (Fig. 2A, $p = 0.093$), or in IAV levels in the nasopharynx or middle ears of adjuvant- and WCV-treated mice.

In contrast to results observed with EF3030, WCV did not reduce PMP1106 density in the middle ears of co-infected mice (Fig. 2B, $p = 0.47$). In 2 of 21 WCV-immunised mice PMP1106

was not detected in either ear, whereas all 18 mice immunised with adjuvant had detectable PMP1106 in at least one ear (Table 1). There were no differences in the proportion of mice displaying bilateral OM (Table 1: 11 of 18 (61%) vs 13 of 21 (62%)), in the median density of PMP1106 in the nasopharynx (Fig. 2B, $p = 0.34$), or in the proportion of mice exhibiting nasopharyngeal loads of less than 10^5 CFU between vaccine groups (4 of 21 (19%) vs 4 of 18 (22%)). Nasopharyngeal IAV loads were comparable between mice administered WCV or adjuvant and co-infected with PMP1106 (Fig. 2D, $p = 0.63$).

Interestingly (Fig. 2), mice had differing IAV loads in the nasopharynx depending on whether they were co-infected with EF3030 or PMP1106. This was the case for both adjuvant-treated mice (EF3030 median 1.88×10^7 IAV genome copies vs PMP1106 median 3.00×10^6 IAV genome copies, $p = 0.0008$, Mann Whitney *U* test) and WCV-treated mice (EF3030 median 7.76×10^6 IAV genome copies vs PMP1106 median 3.35×10^6 IAV genome copies, $p = 0.0169$, Mann *U* Whitney test).

Preliminary histological analysis revealed no significant differences in inflammation of the middle ear between WCV-immunised and adjuvant control groups for mice co-infected with EF3030 (20% vs 50%, $p = 0.69$) or PMP1106 (0% vs 10%, $p = 0.67$).

To investigate whether WCV accelerated the clearance of pneumococci from the middle ears, we compared mice given adjuvant alone with those immunised with WCV 12 days after infection

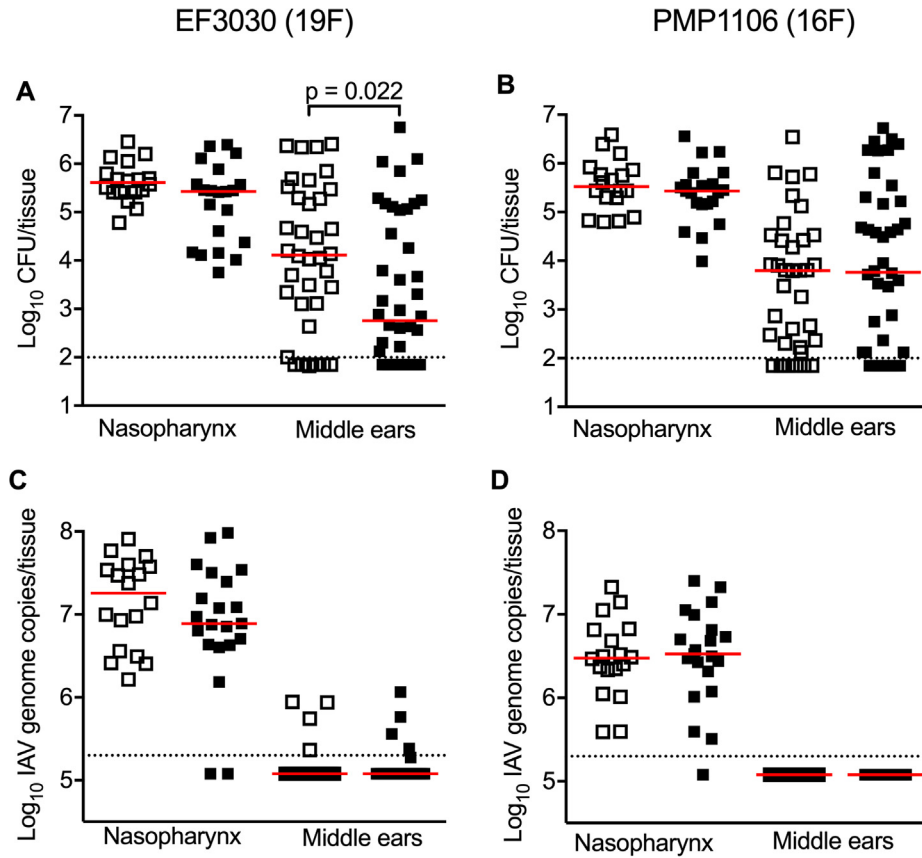


Fig. 2. Effect of WCV on pneumococcal OM six days post-IAV. Mice were administered one dose of either adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected with pneumococci (EF3030, left panel or PMP1106, right panel) and IAV as depicted in Fig. 1A. Pneumococcal (A, B) and IAV (C, D) levels in the nasopharynx and middle ears were determined 6 days post-IAV infection. For the nasopharynx, each data point represents an individual mouse. Counts from each middle ear are represented as individual data points. Figures show data pooled from three independent experiments. Red lines show the median; the dotted line represents the limit of detection (100 CFU for Figures A and B; 1.7×10^5 IAV genome copies for Figures C and D). The Mann Whitney U Test was used to compare control (adjuvant alone) and WCV-treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of WCV on pneumococcal OM type in mice following co-infection.

Pneumococcal isolate	Group	6 days post-IAV				p ^a	12 days post-IAV				p
		OM type					OM type				
		No OM	Unilateral	Bilateral			No OM	Unilateral	Bilateral		
EF3030	Adjuvant	0/18 (0) ^b	5/18 (28)	13/18 (72)	0.167	8/20 (40)	6/20 (30)	6/20 (30)	0.931		
	WCV	4/21 (19)	6/21 (29)	11/21 (52)		11/25 (44)	6/25 (24)	8/25 (32)			
PMP1106	Adjuvant	0/18 (0)	7/18 (39)	11/18 (61)	0.569	8/21 (38)	5/21 (24)	8/21 (38)	0.846		
	WCV	2/21 (10)	6/21 (29)	13/21 (62)		8/18 (44)	5/18 (28)	5/18 (28)			

^a Comparison of adjuvant vs WCV. Fisher's exact test, two-tailed.
^b Data are expressed as number of mice/total mice (%).

with IAV, and found no difference in the number of mice that developed OM (Table 1, EF3030: 14 of 25 (56%) vs 12 of 20 (60%); PMP1106: 13 of 21 (62%) vs 10 of 18 (56%)), nor the levels of pneumococci in their middle ears (Fig. 3A, $p = 0.61$; Fig. 3B, $p = 0.22$). No significant differences were observed between IAV loads in the middle ears of mice administered WCV or adjuvant (Fig. 3C, $p = 1$; Fig. 3D, $p = 0.88$) 12 days post-IAV infection. However, a significant reduction in pneumococcal density was observed in the nasopharynx of mice administered WCV, compared to mice administered adjuvant, for both pneumococcal isolates tested (Fig. 3A, $p = 0.035$; Fig. 3B, $p = 0.011$). This reduction was observed despite comparable levels of IAV in the nasopharynx among vaccine groups (Fig. 3C, $p = 0.34$; Fig. 3D, $p = 0.63$).

We next investigated the immune responses associated with the observed reduction of EF3030 density in the middle ears. Previous studies in adult mice have demonstrated roles for CD4⁺ T cells, and the cytokine IL-17A, in WCV-mediated protection against pneumococcal colonisation [13,27]. We hypothesised that, since the nasopharynx and the middle ear are both mucosal sites, CD4⁺ T cells and IL-17A would be involved in the observed WCV-mediated reduction of EF3030 density in the middle ear. We therefore measured splenic IL-17A responses in vaccinated and co-infected mice (EF3030) 6 days post-IAV infection. *In vitro* stimulation of splenocytes with WCA induced significant production of IL-17A compared to stimulation with PBS (Fig. 4A, $p < 0.001$ for both adjuvant and WCV). In WCA-stimulated samples, we observed a

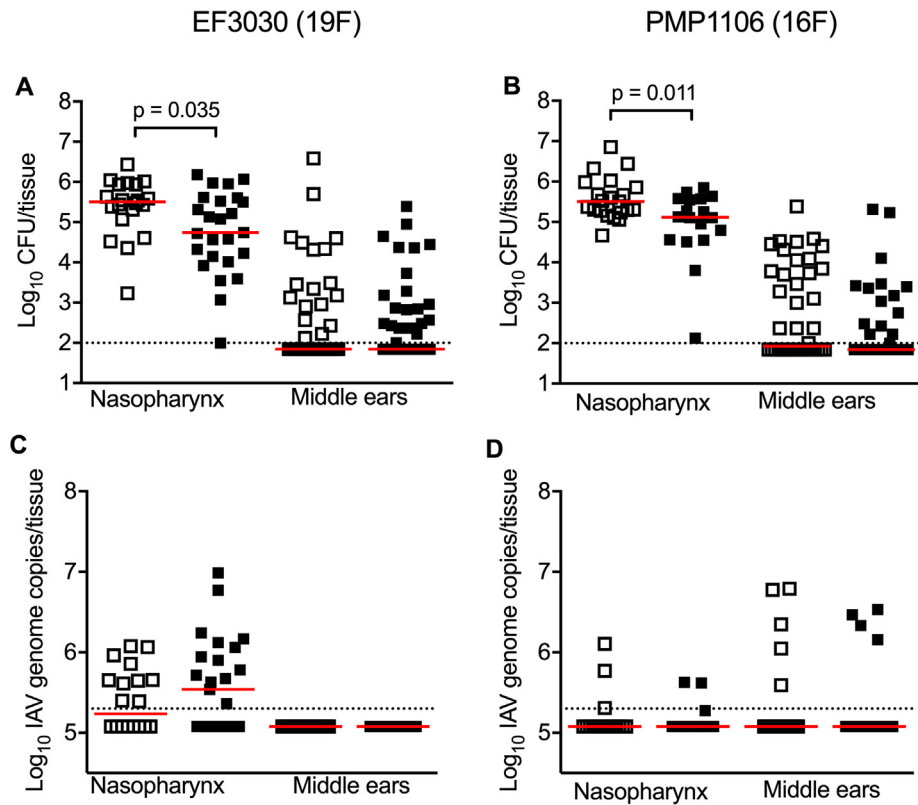


Fig. 3. Effect of WCV on pneumococcal OM 12 days post-IAV. Mice were administered one dose of either adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected with pneumococci (EF3030, left panel or PMP1106, right panel) and IAV as depicted in Fig. 1A. Pneumococcal and IAV levels in the nasopharynx and middle ears were determined 12 days post-IAV infection for mice co-infected with EF3030 (A, C) or PMP1106 (B, D). For the nasopharynx, each data point represents an individual mouse. Counts from each middle ear are represented as individual data points. All graphs show data pooled from three independent experiments. Red lines show the median; the dotted line represents the limit of detection (100 CFU for Figures A and B; 1.7×10^5 IAV genome copies for Figures C and D). The Mann Whitney *U* Test was used to compare control (adjuvant alone) and WCV-treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

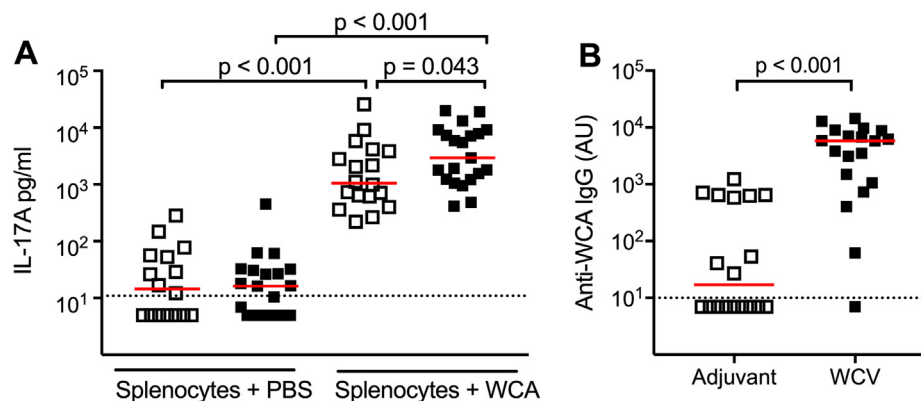


Fig. 4. IL-17A and IgG responses in vaccinated co-infected mice. Mice were administered one dose of adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected (with EF3030 and IAV) as depicted in Fig. 1A. Blood and spleens were harvested 6 days post-IAV infection. WCA-specific IL-17A was measured by ELISA from the supernatants of splenocyte suspensions that had been stimulated with WCA or PBS for 72 h (A). Anti-WCA IgG responses were measured in plasma (B). Each data point represents an individual mouse. All graphs show data pooled from three independent experiments; red lines show the median; the dotted line represents the limit of detection: 10.9 pg/ml of IL-17A, 15.625 AU of IgG, 100 CFU. The Mann Whitney *U* Test was used to compare immune responses in control (adjuvant alone) and WCV-treated mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

small but significant increase in IL-17A responses from mice administered WCV and co-infected with EF3030, compared to the adjuvant-control mice (Fig. 4A, $p = 0.043$).

To investigate the contribution of vaccine-induced antibodies, we measured WCA-specific IgG in the plasma of vaccinated and co-infected mice. As expected, IgG responses were significantly higher in WCV-immunised mice, compared to mice administered adjuvant (Fig. 4B, $p < 0.001$).

We sought to further investigate the role of antibodies and IL-17A in the WCV-mediated reduction of EF3030 in the ears using mice deficient in antibodies and $CD4^+$ T cells. To determine the role of $CD4^+$ T cells in the WCV-induced reduction of EF3030 density in the middle ears, we used the $CD4^+$ T cell-deficient GK1.5 transgenic mouse strain [24]. To investigate the effect of $CD4^+$ T cells only at the time of OM development (i.e. following IAV infection), we depleted WT mice of $CD4^+$ T cells via i.p. administration of the

GK1.5 mAb before (at 17 days old) and after (at 23 days old) IAV infection. Preliminary experiments confirmed that the dosing schedules chosen effectively depleted CD4⁺ T cells at the time of IAV infection, and during the development of IAV-mediated pneumococcal OM, in WT mice (Fig S1). To investigate the role of antibodies in the WCV-mediated reduction of EF3030 in the middle ears, we used antibody-deficient B6.μMT^{-/-} mice. Pneumococcal and IAV co-infection of B6.μMT^{-/-} mice resulted in reduced dissemination of pneumococci to the middle ears compared to co-infection of WT C57BL/6 mice, but dissemination could be increased by providing B6.μMT^{-/-} mice with a source of non-specific antibodies via administration (passive transfer) of naïve C57BL/6 mouse serum [25]. We confirmed that passive transfer of naïve mouse serum to co-infected B6.μMT^{-/-} mice enhanced

pneumococcal density in the middle ears of co-infected B6.μMT^{-/-} mice (Fig S2, p = 0.003) using the infection timeline described in Fig. 1A.

To determine if a single dose of WCV administered at 6 days of age could reduce EF3030 density in the middle ears of co-infected mice in the absence of CD4⁺ T cells and antibody responses, we compared pneumococcal density in the middle ears of vaccinated and co-infected GK1.5 mice, WT mice depleted of CD4⁺ T cells, and B6.μMT^{-/-} mice. WCV immunisation (compared with adjuvant alone) no longer reduced EF3030 density in the middle ears following co-infection in any of these immune-depleted models (Fig. 5B). No significant differences were observed between adjuvant and WCV-immunised immune-deficient mice that developed OM or the proportion of mice exhibiting bilateral OM (Table 2). In addition,

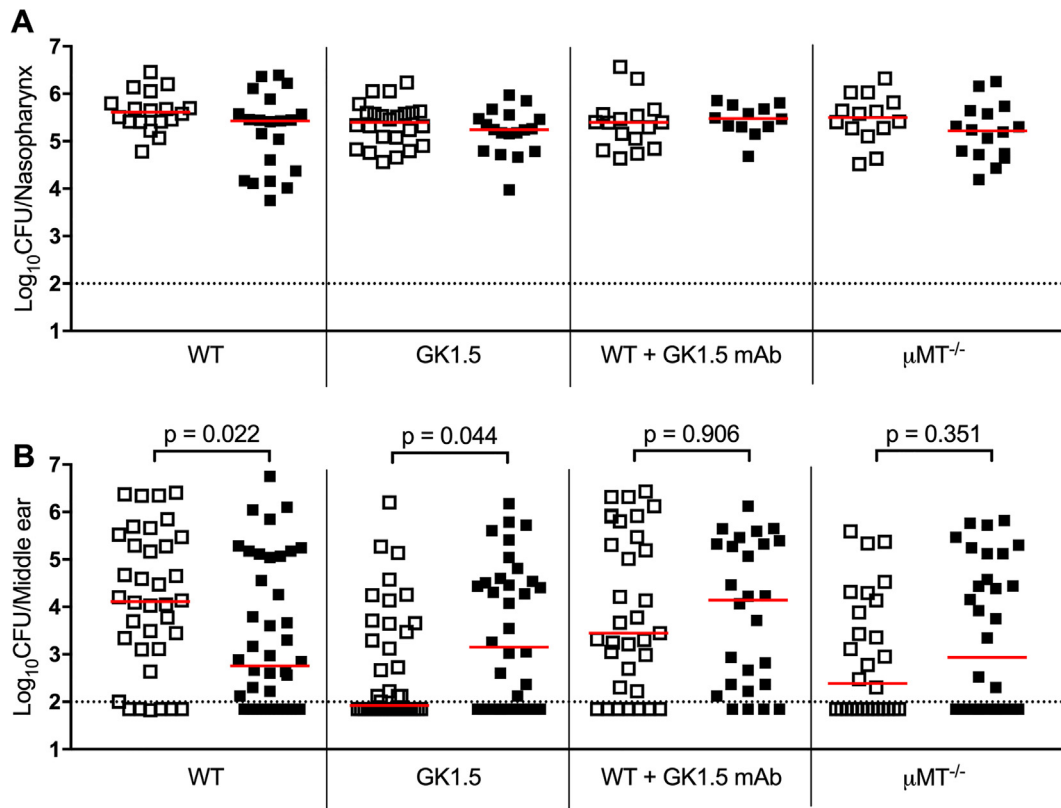


Fig. 5. Effect of CD4⁺ T cell and antibody-depletion on the WCV-mediated impact on pneumococcal density following co-infection with EF3030. C57BL/6 (WT), GK1.5 or B6.μMT^{-/-} mice were administered one dose of adjuvant (open squares) or WCV (closed squares) subcutaneously and then co-infected (with EF3030 and IAV) as depicted in Fig. 1A. CD4⁺ T cells were depleted in WT mice before and after IAV infection (WT + GK1.5 mAb) as described in the methods. B6.μMT^{-/-} mice (μMT^{-/-}) were administered naïve serum as described in the methods. Pneumococcal density was measured in the nasopharynx (A) and middle ears (B) at 6 days post-IAV infection. Each nasopharyngeal data point represents an individual mouse. Counts from each middle ear are represented as individual data points. Red lines show the median; the dotted line represents the limit of detection (100 CFU). All graphs show data pooled from at least two independent experiments. The Mann Whitney U Test was used to compare adjuvant and WCV-treated mice in each experimental condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effect of WCV on EF3030 OM six days post-IAV in immune-deficient mice.

Mouse strain	Group	Number of mice/total mice (%)			p ^a
		No OM	Unilateral	Bilateral	
WT	Adjuvant	0/18 (0)	5/18 (28)	13/18 (72)	0.167
	WCV	4/21 (19)	6/21 (29)	11/21 (52)	
GK1.5	Adjuvant	7/20 (35)	5/20 (25)	8/20 (40)	0.386
	WCV	3/18 (17)	7/18 (39)	8/18 (44)	
WT + GK1.5 mAb	Adjuvant	2/16 (13)	2/16 (13)	12/16 (75)	0.613
	WCV	1/12 (8)	0/12 (0)	11/12 (92)	
μMT ^{-/-}	Adjuvant	4/14 (29)	4/14 (29)	6/14 (43)	0.904
	WCV	4/16 (19)	6/16 (38)	6/16 (38)	

^a Comparison of adjuvant vs WCV, Fisher's Exact test.

tion, no significant differences in pneumococcal density in the nasopharynx were observed between adjuvant and WCV-treated mice in each model (Fig. 5A, $p > 0.05$ for all). Taken together, these results suggest that both CD4⁺ T cells and antibodies are involved in the WCV-mediated reduction in EF3030 density observed in WT co-infected mice.

4. Discussion

Our results demonstrate that although WCV significantly reduced pneumococcal nasopharyngeal density following IAV infection, it did not prevent the spread of pneumococci from the nasopharynx to the middle ear, nor did it enhance clearance of OM in our model. WCV significantly reduced pneumococcal density in the middle ears of co-infected mice at the peak of OM in one of two strains tested. Experiments in immune-deficient/depleted mice indicate that both CD4⁺ T cells and antibodies were involved in the WCV-mediated reduction of pneumococcal isolate EF3030 density in the middle ears.

There are multiple factors that may explain why WCV was unable to prevent the development of OM in this model. These include deficiencies in the immune response of infants to vaccination [29] and pneumococcal infection [30], the inability to provide more than one immunisation prior to infection, the limited time available for mice to mount protective immunity before the induction of OM, and the magnitude of pneumococcal disease induced following co-infection, evidenced by almost 100% of co-infected mice developing pneumococcal OM (Fig. 1). Mechanistically, i.n. IAV infection induces inflammation in the nasopharynx which leads to a significant increase in pneumococcal colonisation density and duration at this site [17,31]. In addition, IAV can disperse pneumococcal nasopharyngeal biofilms to release virulent bacteria with a greater propensity to infect the middle ear [32]. To prevent the development of OM in this model, WCV-induced immunity would need to control pneumococcal colonisation density and prevent the bacteria from reaching the middle ear, or to mount a rapid immune response in the middle ear to clear disseminating pneumococci. The former scenario was observed in 4 of 21 and 2 of 21 mice administered WCV and infected with EF3030 and PMP1106, respectively, compared to none of 18 mice administered adjuvant alone (Table 1). The observed reduction of EF3030 density at 6 days post-IAV suggests that WCV temporarily achieved the latter scenario, but this was not observed for PMP1106. However, given that there was no reduction in EF3030 density in the middle ear at a later time point (Fig. 2A), our data demonstrate that WCV did not enhance the overall clearance of this isolate from the middle ears. The clinical relevance of the reduced density of EF3030 observed during peak OM is unknown, and histology did not demonstrate a reduction in inflammation in WCV-immunised mice. Hearing tests were not conducted as part of this study, but given that levels of EF3030 in the middle ears of WCV-immunised mice were comparable to those that have previously been associated with hearing impairment following co-infection in this model [16], we do not expect that the WCV-mediated reduction in density to translate to improved hearing in vaccinated mice.

Studies of pneumococcal OM in mice have demonstrated that inflammatory mediators including neutrophil-recruiting IL-17A [33] and myeloperoxidase [34], as well as TLR2 signalling and neutrophil and macrophage recruitment [35], are important in clearing pneumococci from the middle ear, while T and B cells migrate to and proliferate at this site several days after infection [36,37]. The role of antibodies in clearing pneumococcal OM remains uncertain. Clinical evidence from small cohorts suggest that poor local and systemic antibody responses to pneumococcal antigens are associated with recurrent OM and upper respiratory tract infec-

tions in children [38,39], but larger studies in high-risk populations have not confirmed this association [40]. In an experimental co-infection setting, antibodies, along with neutrophil extracellular traps, can facilitate pneumococcal multiplication in the middle ear [25]. WCV-mediated protection against pneumococcal colonisation requires IL-17A but not antibodies, whereas protection against invasive disease is antibody-mediated [9,41]. As expected, we observed significantly enhanced systemic IL-17A and antibody responses in WCV-immunised mice co-infected with EF3030 compared to mice administered adjuvant alone (Fig. 4A, D). It is possible that the WCV-mediated reduction in EF3030 middle ear density was dependent on immune responses that took place earlier than 6 days post-IAV. Alternatively, higher levels of WCV-induced antibodies and CD4⁺ T cells (the major subset responsible for IL-17A production in splenocytes following *in vitro* stimulation by WCA [13]) may have migrated to the site of infection. Although we did not measure IL-17A or antibodies in the middle ear, other studies have demonstrated successful extravasation of vaccine-specific antibodies to the middle ear following parenteral immunisation [42]. It is possible that increased IL-17A and antibody responses in the middle ear may have initially reduced EF3030 density in WCV-immunised mice. Our data demonstrating that WCV could no longer reduce EF3030 density in mice deficient of CD4⁺ T cells and antibody responses (Fig. 5B) support this hypothesis. This is also consistent with data from preclinical evaluation of other investigational pneumococcal vaccines. For example, Rosch et al. [43] demonstrated that CD4⁺ T cells at the time of vaccination were required to prevent OM following pneumococcal challenge (with or without IAV co-infection) for both a live attenuated pneumococcal vaccine (BHN97ΔftsY) delivered i.n., and for i.p. vaccination with PCV13. These authors also found that protection was associated with CD4⁺ T cell-dependent immunoglobulin isotype switching (mouse IgG2a and 2c for BHN97ΔftsY, and IgG1 for PCV13) [43]. Additionally, co-infected mice deficient in IL-17A receptor signalling had higher pneumococcal loads in the middle ears compared to co-infected WT mice following i.n. immunisation with recombinant PspA and cholera toxin [44]. A role for T_H17 responses in preventing OM is also supported by findings in a cohort of otitis-prone children in New York. The definition of stringently otitis-prone (sOP) was assigned to children experiencing three or more OM episodes within a 6- to 12-month period by Pichichero and colleagues [45], who examined OM episodes using tympanocentesis in 840 children over 10 years. Investigations of immune responses in this population demonstrated that sOP children showed reduced production of IL-17A by peripheral blood memory T cells after stimulation with heat-killed pneumococci or pneumococcal protein antigens compared to non-sOP children [46,47], suggesting that reduced CD4⁺ T cell-mediated IL-17A responses were associated with an increased risk of recurrent OM.

Antigenic differences between the pneumococcal challenge strains tested here and the WCV strain, RM200, may have accounted for the lack of protection observed in our model, and explain why WCV reduced EF3030 but not PMP1106 density in the middle ears of co-infected mice. Previous studies have investigated the ability of WCV-induced immune responses to bind to and kill different strains and serotypes of pneumococci *in vitro*. Using ELISA and flow cytometry, Moffitt et al. [21] demonstrated that WCV-specific antisera bound to a range of pneumococci (representing 12 serotypes and at least 10 MLST types, and including both invasive and carriage isolates), and 20 isogenic mutants of TIGR4 expressing different capsule polysaccharides, at significantly higher levels compared to adjuvant-specific antisera. However, the fold-increase in binding was variable between strains. Less strain variation was observed when IL-17A responses were detected following stimulation of WCV-immunised splenocytes with different pneumococcal strains (26–43-fold difference in expression [21]),

and when WCV-specific antibodies were tested for their ability to induce phagocytosis of pneumococcal strains by mouse peritoneal cells *in vitro* (12 to 39% killing [48]).

Pneumococcal strains can vary greatly at the genetic level in a way that gives rise to considerable variation in pneumococcal surface proteins, which are the targets of the WCV-response. For example, of 616 pneumococcal carriage isolates, Croucher et al. [49] identified 39 and 59 variants of pneumococcal surface proteins A (PspA) and C (PspC), respectively. Genetic comparison of RM200, EF3030 and PMP1106 could show if this is due to differences in the structure of antigens in all three strains. For example, differences in PspA may prevent or reduce WCV-induced anti-PspA from binding to PMP1106 and reducing IAV-induced OM [50].

Differences between the two pneumococcal strains may have also affected the host response to IAV in our model: mice co-infected with EF3030 had higher levels of IAV in the nasopharynx compared with mice co-infected with PMP1106. Diavatopoulos et al. observed that mice had reduced IAV loads in the presence of pneumococci [51]. More recently, Ortigoza et al. demonstrated that pneumococcal neuraminidases (NanA and NanB) reduce IAV acquisition and shedding [52]. It is possible that differences in sialidase expression by pneumococcal strain may have influenced IAV levels in our model.

Although WCV did not affect the density of nasopharyngeal colonisation 6 days post-IAV (Fig. 2A and B), it significantly reduced colonisation density at 12 days post-IAV for both pneumococcal strains tested (Fig. 3A and B). This is consistent with the broad efficacy of WCV in adult and infant mice [9,10,13,29]. The enhanced nasopharyngeal clearance observed in WCV-immunised mice may translate to protection against future episodes of pneumococcal OM following additional IAV infections. A second IAV challenge in vaccinated and co-infected mice that remain colonised with pneumococci would evaluate whether WCV protection against IAV-induced OM increased with time. High pneumococcal carriage density is associated with the development of pneumococcal disease [53–55] and transmission [56,57]. Therefore, WCV-mediated reductions in carriage may protect against pneumococcal disease and transmission.

In conclusion, this study was the first to test the effect of WCV on IAV-induced pneumococcal disease. WCV administration did not prevent the development of pneumococcal OM, nor speed up the resolution of infection. However, a strain-dependent reduction in density of pneumococci in the middle ears was observed. In addition, WCV reduced the nasopharyngeal density of both isolates tested. Overall, our data suggest that WCV may not directly impact on IAV-induced OM within an individual, but may have a population-level effect due to enhanced clearance of pneumococcal colonisation.

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Authors contributions

JM, EMD, RAB, JMO, JEKM, RRB, RM, OLW and CS participated in study design, and data analysis and interpretation. NW performed FACS analysis and JSP conducted the histological assessment of

level of inflammation in middle ear tissue sections. All other experiments were performed by JM. JM drafted the manuscript with CS and OLW. All authors participated in editing of the manuscript and approved of the final manuscript.

Conflict of interest

OLW, CS, EMD and JM are recipients of the Robert Austrian Award in Pneumococcal Vaccinology, which is funded by Pfizer but awarded by ISPPD. RM is a scientific founder, consultant, and equity owner at Affinivax, a biotechnology company based in Cambridge MA that is devoted to the development of vaccines for developing and developed countries. EKM is a member of the PATH Pneumococcal Vaccine Project wSP Scientific Advisory Board. The other authors have no conflicts of interest to declare.

Declaration of interests

The authors declare that they have no known competing financial interests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.03.013>.

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