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Adjuvant system AS02$_V$ enhances humoral and cellular immune responses to pneumococcal protein PhtD vaccine in healthy young and older adults: Randomised, controlled trials☆

Isabel Leroux-Roels[a,*], Jeanne-Marie Devaster[b], Geert Leroux-Roels[a], Vincent Verlant[b], Isabelle Henckaerts[b], Philippe Moris[b], Philippe Hermand[b], Pascale Van Belle[b], Jan T. Poolman[b,c], Pierre Vandepapelière[b,d], Yves Horsmans[e]

[a] Centre for Vaccinology, Ghent University and Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium
[b] GlaxoSmithKline Vaccines, Rixensart, Belgium
[c] Crucell, PO Box 2048, 2301 CA, Leiden, The Netherlands
[d] Neovacs S.A., 3-5, Impasse Reille, 75014 Paris, France
[e] Unité de Pharmacologie Clinique, University Hospital St-Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium

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ABSTRACT

Background: The protection elicited by polysaccharide pneumococcal vaccines against community-acquired pneumonia in older adults remains debatable. Alternative vaccine targets include well-conserved pneumococcal protein antigens, such as pneumococcal histidine triad protein D (PhtD).
Objective: To evaluate humoral and cellular immune responses and safety/reactogenicity following immunisation with PhtD vaccine with or without adjuvant (alum or AS02$_V$) in older (≥65 years) and young (18–45 years) healthy adults.
Methods: Two phase I/II, single-blind, parallel-group studies were conducted in 150 older and 147 young adults. Participants were randomised to receive 2 doses (months 0 and 2) of PhtD 30 μg, PhtD 10 μg plus alum, PhtD 30 μg plus alum, PhtD 10 μg plus AS02$_V$ or PhtD 30 μg plus AS02$_V$, or the 23-valent polysaccharide pneumococcal vaccine (23PPV) at month 0 with placebo (saline solution) at month 2. Safety/reactogenicity was assessed. PhtD-specific antibody, T cell and memory B cell responses were evaluated.
Results: Solicited adverse events were more common in young participants and with adjuvanted vaccines. No vaccine-related serious adverse events were reported. Although anti-PhtD geometric mean antibody concentrations (GMCs) were consistently lower in the older adult cohort than in young adults, GMCs in the older cohort following PhtD 30 μg plus AS02$_V$ were comparable to those induced by plain PhtD or PhtD 30 μg plus alum in the young cohort. Compared with alum adjuvant, AS02$_V$ adjuvant system was associated with an increased frequency of PhtD-specific CD4 cells in both cohorts and a significantly higher specific memory B cell response in the older cohort, similar to responses obtained in the young cohort.
Conclusion: The improved immune response to PhtD vaccine containing the AS02$_V$ adjuvant system in comparison to alum suggests that the reduced immune response to vaccines in older adults can be partially restored to the response level observed in young adults. ClinicalTrials.gov identifiers: NCT00307528/NCT01767402.

Abbreviations: AE, adverse event; AS, adjuvant system; ATP, according-to-protocol; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; GMC, geometric mean antibody concentration; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIV, human immunodeficiency virus; IFN, interferon; IL, interleukin; MPL, 3-*O*-desacyl-4′monophosphoryl lipid A; PCV, pneumococcal conjugate vaccine; PhtD, pneumococcal histidine triad protein D; 23PPV, 23-valent polysaccharide pneumococcal vaccine; QS21, *Quillaja saponaria* Molina fraction 21; TNF, tumour necrosis factor.

* Corresponding author. Tel.: +32 9 332 34 22; fax: +32 332 63 11.
E-mail addresses: isabel.lerouxroels@ugent.be (I. Leroux-Roels), jeanne-marie.devaster@gsk.com (J.-M. Devaster), geert.lerouxroels@ugent.be (G. Leroux-Roels), vincent.verlant@gsk.com (V. Verlant), isabelle.henckaerts@gsk.com (I. Henckaerts), philippe.moris@gsk.com (P. Moris), philippe.hermand@gsk.com (P. Hermand), pascale.van-belle@gsk.com (P. Van Belle), jan.poolman@crucell.com (J.T. Poolman), pierre.vandepapeliere@hotmail.com (P. Vandepapelière), yves.horsmans@uclouvain.be (Y. Horsmans).

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1. Introduction

It is widely accepted that 'immunosenescence', the deterioration of immune function with advancing age, also contributes to the increased incidence of morbidity and mortality from infectious diseases among older adults [1] and reduced responses to vaccination [2–4]. The 23-valent polysaccharide pneumococcal vaccine (23PPV) is recommended for use in older adults in most countries but, while there is evidence for a significant reduction in the risk of pneumococcal bacteraemia, protection against non-bacteraemic pneumonia in this population has been a matter of debate [5–9]. Being T cell independent, polysaccharide antigens also fail to induce adequate immunological memory and improved responses upon revaccination [10]. Polysaccharide pneumococcal conjugate vaccines (PCVs) elicit a better priming response in adults [11] but their impact on clinical respiratory outcomes in this population remains to be established. Also, the introduction of PCVs into paediatric vaccination programmes led to a decrease in vaccine serotype pneumococcal disease in adults, which may limit the benefit of PCVs with same serotype composition in older age groups [9,12].

Common pneumococcal proteins are interesting candidate antigens for new pneumococcal vaccines, since they offer the potential for serotype-independent coverage through either antibody or cellular responses [13–18]. The pneumococcal histidine triad protein D (PhtD) is one such conserved pneumococcal protein antigen that has been shown to elicit functional antibodies [19–21] and provide protection against pneumonia in animal models [20,22]. The need to improve immune responses in older adults has also led to the search for vaccine adjuvants that enhance the magnitude and quality of antigen-specific immune response [23]. $AS02_V$ is an adjuvant system that consists of an oil-in-water emulsion combined with two potent immunostimulants, 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) and the saponin QS21 (*Quillaja saponaria* Molina, fraction 21; Antigenics Inc., a wholly owned subsidiary of Agenus Inc., Lexington, MA, USA) [24].

Two studies were conducted to examine the immunogenicity and reactogenicity of two doses of PhtD vaccine administered with or without $AS02_V$ or alum adjuvant in healthy adults. The studies were of identical design, apart from the age of participants: one study included adults aged 65 years or older, while the other included younger adults.

2. Materials and methods

2.1. Study design and participants

In the phase I/II, single-blind, parallel-group studies, older (≥65 years) and young (18–45 years) adults in generally good health were eligible for inclusion. Major exclusion criteria included previous vaccination against *Streptococcus pneumoniae* or with a MPL/QS21-containing vaccine, administration of immune-modifying drugs within the last 6 months, and bacterial pneumonia within 3 years of the first dose. A full list of inclusion/exclusion criteria is provided in Appendices A and B.

Using a centralised randomisation system on the internet, participants were randomised (1:1:1:1:1:1:1) by the investigator at each site to receive vaccination at 0 and 2 months with PhtD 30 μg, PhtD 10 μg plus alum (aluminium phosphate, $AlPO_4$), PhtD 30 μg plus alum, PhtD 10 μg plus $AS02_V$ or PhtD 30 μg plus $AS02_V$, or 23PPV (*Pneumovax*™; Sanofi Pasteur MSD, Lyon, France) at month 0 followed by placebo (saline solution) at month 2 (control group). The studies were single-blind, meaning that participants were unaware of the vaccination assignment.

Co-primary objectives of the studies were to evaluate the safety, reactogenicity and immunogenicity of plain or adjuvanted PhtD, with evaluation of the persistence and quality of the antibody response, and cellular immune response as secondary objectives. The young cohort study was conducted at the Catholic University of Louvain, Brussels, Belgium, between October 2003 and November 2004 and the older cohort study was conducted at the Centre for Vaccinology, Ghent University Hospital, Belgium, between January 2004 and March 2005 followed by additional 2-year follow-up; 1-year results are presented for both cohorts. All participants gave written informed consent to the studies, which were approved by local ethics committees and conducted in accordance with the Declaration of Helsinki.

2.2. Vaccines

The PhtD vaccine was supplied as freeze-dried pellets in monodose vials to be reconstituted with phosphate buffered saline or with adjuvant formulation $AS02_V$ liquid in pre-filled syringes, or was supplied adsorbed on alum as a liquid in monodose vials. 23PPV was supplied in pre-filled syringes and placebo saline in monodose vials. Influenza vaccine (*Fluarix*™; GlaxoSmithKline, Rixensart, Belgium) was offered free of charge to the older cohort during the study. All study vaccines were administered by intramuscular injection (0.5 ml) into the deltoid region of the upper right arm. Participants were observed closely for at least 30 min after vaccination.

2.3. Reactogenicity and safety assessment

Solicited local (injection site pain, redness and swelling) and general (fatigue, fever, gastrointestinal symptoms, headache, malaise, myalgia and sweating) adverse events (AEs) were recorded by the participants on diary cards during the 7-day follow-up after each vaccination. Unsolicited AEs were recorded within 30 days after each vaccination. Serious AEs were reported throughout the study. Duration, causality and outcome of AEs were recorded. All solicited local reactions were considered causally related to vaccination; the relationship of other AEs was classified as possible or not causally related. AE intensity was scored on a scale from 1 to 3. Grade 3 AEs were defined as preventing normal daily activity, apart from grade 3 solicited fever, which was defined as oral/axillary temperature >39.0 °C, and grade 3 solicited swelling or redness, defined as diameter >50 mm. Grade 2 pain was defined as painful when the limb was moved.

2.4. Immunogenicity assays

In both cohorts, blood samples were taken within 14 days before the first vaccination and at post-vaccination intervals up to 10 months after the second vaccine dose. All blood samples were processed and stored appropriately until analysed using in-house methods at the laboratories of GlaxoSmithKline, Rixensart, Belgium.

Anti-PhtD antibody concentrations were measured at months 0 (before the first vaccine dose), 1, 2 (before the second dose), 3 and 12 using an enzyme-linked immunosorbent assay (ELISA; assay cut-off 0.04 μg/ml), as described previously [22]. A passive transfer mouse model assay was used to evaluate the protection provided by anti-PhtD antibodies in pooled sera collected at months 0 and 3 from participants who received PhtD 30 μg, PhtD 30 μg plus alum or PhtD 30 μg plus $AS02_V$. Sera from each vaccine group were administered intraperitoneally into OF1 mice 1 h before intranasal challenge with a lethal dose (10^5 cfu) of *S. pneumoniae* serotype 3. Mortality induced by infection was monitored for 10 days. A rabbit anti-PhtD antiserum, generated in-house using recombinant PhtD as immunogen, was the positive control in each experiment.

In the groups that received PhtD 30 μg plus alum or PhtD 30 μg plus AS02$_V$, the frequencies of PhtD-specific IgG-producing memory B cells induced after vaccination were assessed by B cell Elispot [25] after in vitro differentiation for 5 days. The frequencies of PhtD-specific CD4 or CD8 T cells induced after vaccination were determined by intracellular cytokine flow cytometry and were identified as CD4$^+$/CD8$^+$ T cells producing one or more markers among interferon (IFN)-γ, interleukin (IL)-2, IL-4 and/or tumour necrosis factor (TNF)-α for the first intracellular staining and among CD40L, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2 and/or TNF-α for the second intracellular staining upon short term in vitro stimulation with PhtD overnight for 18 h. Choice of markers was based on practicability and knowledge of their roles in the vaccine-induced cell-mediated immune response. IFN-γ is a critical cytokine for innate and adaptive immunity and characterises Th1 T cells [26], while IL-2 is a cytokine associated with memory T cells and T cell proliferation and differentiation [27] and IL-4 characterises Th2 T cells [28]. TNF-α is a pro-inflammatory cytokine that is also a neutrophil chemoattractant [29] and CD40L, which is a co-stimulatory ligand required for T cell help that also induces the differentiation of B cells [30,31] and, as it is expressed on activated CD4$^+$ cells, allows the sensitivity of the intracellular staining assay to be improved [32,33]. GM-CSF stimulates the growth and differentiation of haematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils, and erythrocytes, and has the capacity to increase antigen-induced immune responses and alter the Th1/Th2 cytokine balance [34].

Intracellular cytokine flow cytometry methods were as described by Maecker et al. [35] and Moris et al. [36], except that in vitro stimulation was performed using a pool of 20-mer peptides (1.25 μg/ml) that overlapped by 10 amino acids and covered the sequence of the relevant antigens. Anti-CD4-PerCP and anti-CD8-APC-Cy7 were used for extracellular staining, and for intracellular staining, either anti-CD40L-FITC, anti-IL-2-APC, anti-GM-CSF-PE and anti-TNF-PE-Cy7 or BD Simultest^{TM} anti-IFN-γ-FITC/anti-IL-4-PE, anti-IL-2-APC and anti-TNF-α-PE were used. Representative gating strategy for flow cytometry is shown in Appendices A and B. The frequencies of CD4$^+$ T cells expressing each marker after in vitro stimulation are presented.

2.5. Statistical analyses

In each study, 92 subjects (23 per group) were required to assess adjuvant type (alum or AS02$_V$) and antigen concentration (PhtD 10 μg or 30 μg). The assumed standard deviation was 0.5 and considered effect size was 0.4 to achieve >90% power with an *F* test of each factor at 5% significance level. Two groups of identical sizes were added as controls. Therefore, the target sample size for the two studies combined was 300 enrolled subjects (25 per group) in order to achieve 276 evaluable subjects (23 per group).

All evaluable participants (i.e. those meeting all eligibility criteria, complying with the procedures defined in the protocol, with no elimination criteria during the study, for whom immunogenicity data were available) were included in the according-to-protocol (ATP) cohort for analysis of immunogenicity. Anti-PhtD geometric mean antibody concentrations (GMCs) were calculated with 95% confidence intervals (CIs). The anti-PhtD antibody response one month after each vaccine administration was compared between groups using a two-way ANOVA model on log10 transformed data. The model included the antigen dose (PhtD 10 μg or 30 μg), type of adjuvant (alum or AS02$_V$) and antigen dose by type of adjuvant interaction as factors.

The frequencies of CD4/CD8 T cells that produced cytokines, and the frequencies of PhtD-specific plasma cells generated by in vitro cultured memory B cells, were compared at month 3 between the PhtD 30 μg plus alum and PhtD 30 μg plus AS02$_V$ groups using the Wilcoxon rank-sum test. In the antibody passive transfer assay, time to death in mice was estimated non-parametrically by the Kaplan–Meier method and was calculated from day 0 (day of transfer) to the day of death or censoring at day 10. The passive transfer protection data presented are qualitative only as the model used does not have the capacity to show differences between vaccines. The safety analysis was conducted on the total vaccinated cohort.

3. Results

3.1. Study population

A total of 147 and 150 subjects were enrolled and vaccinated in the young adult and older adult studies, respectively, and 144 and 147 participants, respectively, were included in the ATP cohort for immunogenicity. Reasons for exclusion from the ATP cohort for immunogenicity and withdrawals are provided in Appendices A and B. The demographic characteristics of all groups were comparable with respect to median age and ethnic origin, while the female to male ratio varied (Table 1).

3.2. Reactogenicity and safety

Overall, solicited local and general AEs were more common in the young cohort than in the older cohort (Fig. 1). Reactogenicity tended to be higher in the AS02$_V$ groups than in the other groups but, in all groups that received adjuvanted vaccine, there was no consistent evidence to suggest greater reactogenicity with PhtD 30 μg versus the 10 μg dose.

Pain was the most common solicited local AE in both young and older participants and its incidence was highest with PhtD plus AS02$_V$ in both groups (Fig. 1). In the young cohort, after the first dose, 13, 2, 5, 11, 15 and 18 subjects reported grade 2 (maximum severity over the solicited period) injection site pain in the 23PPV, PhtD 30 μg, PhtD 10 μg plus alum, PhtD 30 μg plus alum, PhtD 10 μg plus AS02$_V$ and PhtD 30 μg plus AS02$_V$ groups, respectively. All grade 2 pain lasted a maximum of two consecutive days except one report in the PhtD 30 μg plus AS02$_V$ group, which lasted three days. Grade 3 pain was reported by four subjects in the 23PPV group, four in the PhtD 10 μg plus AS02$_V$ group and one subject in the PhtD 30 μg plus AS02$_V$ group. All grade 3 pain lasted a maximum of two consecutive days. After the second dose, 0, 1, 3, 9, 11 and 19 subjects reported grade 2 injection site pain in the 23PPV (placebo as second injection), PhtD 30 μg, PhtD 10 μg plus alum, PhtD 30 μg plus alum, PhtD 10 μg plus AS02$_V$ and PhtD 30 μg plus AS02$_V$ groups, respectively. All grade 2 pain lasted a maximum of two consecutive days, apart from five consecutive days for one subject in the PhtD 10 μg plus alum group, and three and four consecutive days for two subjects in the PhtD 30 μg plus AS02$_V$ group. Grade 3 pain was reported by seven subjects in the PhtD 10 μg plus AS02$_V$ group and by two in the PhtD 30 μg plus AS02$_V$ group. All grade 3 pain lasted a maximum of two consecutive days.

In the older cohort, up to eight subjects in each study group reported grade 2 injection site pain after the first dose and up to seven reported grade 2 pain after the second dose, with the highest incidences in the AS02$_V$ groups. All grade 2 pain lasted a maximum of two consecutive days, except for grade 2 pain in two subjects in the PhtD 10 μg plus alum group (three consecutive days post-dose 1 or seven days post-dose 2) and one subject in the PhtD 30 μg plus AS02$_V$ group (three days post-dose 2). No grade 3 pain was reported in the older cohort.

In the young cohort, there were few reports of grade 3 redness or swelling (≤3 reports of each grade 3 event in each group). In older participants, there was one report each of grade 3 redness

Table 1
Demographics of participants (ATP cohorts for immunogenicity).

Group	23PPV	PhtD 30 μg	PhtD 10 μg + alum	PhtD 30 μg + alum	PhtD 10 μg + AS02$_V$	PhtD 30 μg + AS02$_V$
Young adult cohort (18–45 years), *n*	24	24	23	25	23	25
Median age, years	27.5	25.0	27.0	27.0	26.0	25.0
Age range, years	18–43	19–44	19–45	18–45	18–44	19–39
Gender (%), female/male	62.5/37.5	29.2/70.8	52.2/47.8	32.0/68.0	52.2/47.8	52.0/48.0
Ethnicity (%)						
Caucasian	100	91.7	87.0	96.0	91.3	96.0
Older adult cohort (≥65 years), *n*	25	25	24	25	25	23
Median age, years	73.0	72.0	72.5	73.0	69.0	70.0
Age range, years	65–83	66–80	66–84	65–82	65–78	65–80
Gender (%), female/male	52.0/48.0	40.0/60.0	37.5/62.5	44.0/56.0	24.0/76.0	43.5/56.5
Ethnicity (%)						
Caucasian	100	100	100	100	100	100

following 23PPV and grade 3 swelling following PhtD 30 μg plus AS02$_V$.

Fatigue and headache were the most commonly reported solicited general symptoms in both cohorts. There were no reports of grade 3 solicited general symptoms in the older cohort, apart from one report of grade 3 malaise in the PhtD 10 μg plus AS02$_V$ group. There was one report of fever (38.4 °C) in the PhtD 30 μg plus AS02$_V$ group of the older cohort that lasted one day (on day 5 post-dose 2). In the young cohort, one to five subjects in each study group (maximum in PhtD 30 μg plus AS02$_V$ group) reported fever after vaccination that lasted for a maximum of two consecutive days and occurred mainly on day 0 and/or day 1 after vaccination. All but two were <38.5 °C, with no reports of grade 3 fever (>39 °C). Grade 3 fatigue followed 2.0%, 4.3% and 8.2% of PhtD 10 μg plus alum, PhtD 10 μg plus AS02$_V$ and PhtD 30 μg plus AS02$_V$ doses, respectively, while reports of other grade 3 solicited general AEs were infrequent (≤3 reports of each grade 3 event in each group).

The overall incidence of unsolicited AEs was comparable between groups for both cohorts (data in Appendices A and B). There were no reports of grade 3 unsolicited AEs causally related to vaccination in the older cohort and one report each of grade 3 'feeling cold' and tremor possibly related to vaccination in the young cohort (both in PhtD 30 μg plus AS02$_V$ group). No vaccine-related serious AEs were reported during the study.

3.3. Humoral immunity

At all time points, all participants in both cohorts were seropositive for antibodies against PhtD (cut-off 0.04 μg/ml). Anti-PhtD GMCs were consistently lower in the older cohort than in the young

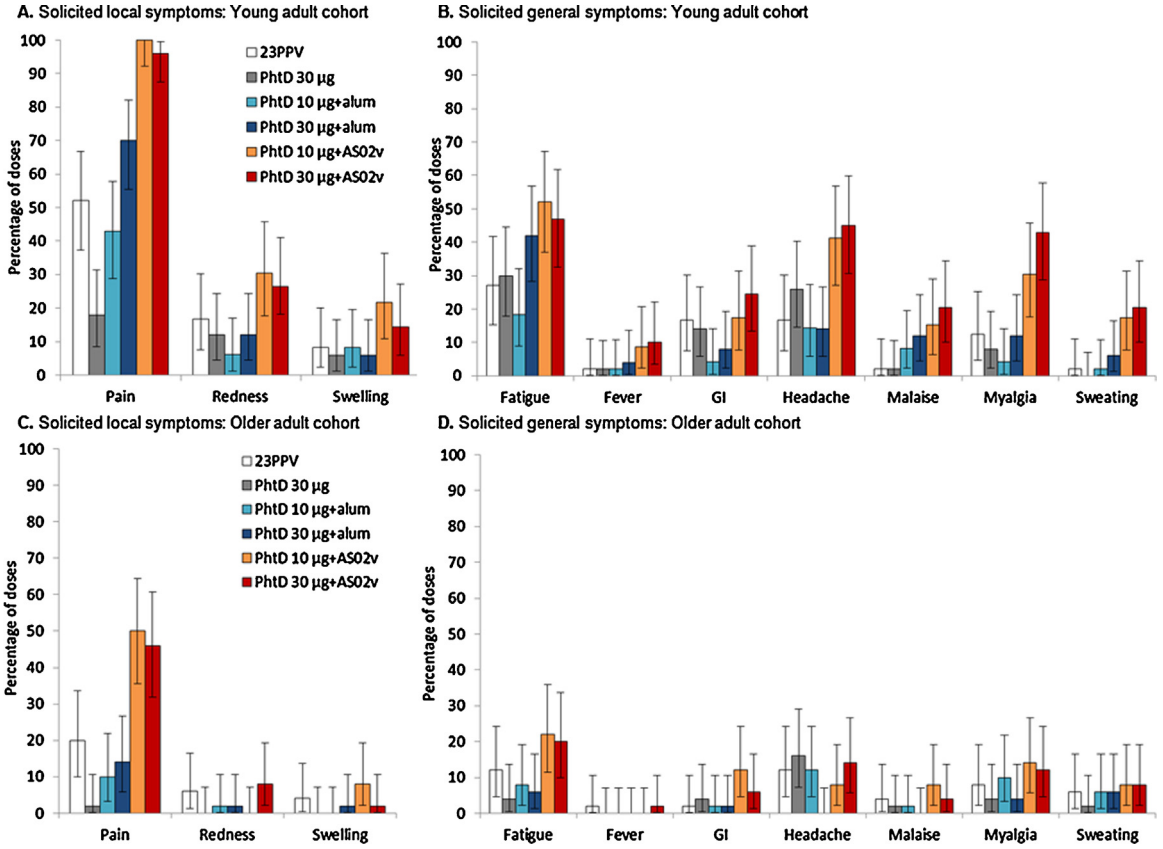

Fig. 1. Overall per dose incidence (with 95% confidence intervals) of solicited local ((A) and (C)) and general ((B) and (D)) symptoms within the 7 day follow-up after each vaccine dose in young ((A) and (B)) and older ((C) and (D)) adult participants (total vaccinated cohorts). 23PPV, 23-valent polysaccharide pneumococcal vaccine. All other groups were administered PhtD (30 μg or 10 μg) with or without adjuvant (AS02$_v$ or alum). GI, gastrointestinal.

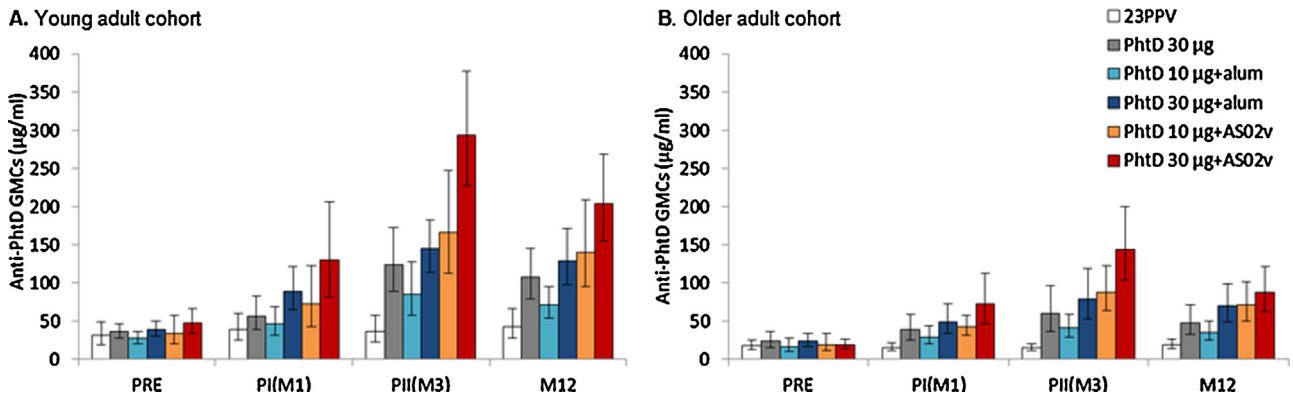

Fig. 2. Anti-PhtD antibody geometric mean concentrations (GMCs with 95% confidence intervals) in the young (A) and older (B) cohorts before (PRE), one month after the first (M1) and second vaccine doses (M3) and ten months after second dose (M12) (ATP cohorts for immunogenicity).

cohort before vaccination and after each vaccine dose; after the first dose, GMC responses in the young cohort were similar to those after the second dose in the older cohort (Fig. 2). In the older cohort, the anti-PhtD GMC after the second dose of PhtD 30 μg plus $AS02_V$ (143.7 μg/ml [95% CI 103.5; 199.5]) was comparable to that in the young cohort after the second dose of plain PhtD 30 μg or PhtD 30 μg plus alum (124.2 μg/ml [95% CI 89.3; 172.8]) and 144.8 μg/ml [95% CI 114.5; 183.2], respectively) (Fig. 2).

In both cohorts, no statistically significant interactions between antigen dose and adjuvant were detected in the two-way ANOVA models, indicating that the dose effect (10 μg versus 30 μg PhtD) did not depend on the adjuvant ($AS02_V$ or alum) and vice versa. The ANOVA model showed a statistically significant dose effect, with a higher anti-PhtD antibody response with PhtD 30 μg than with PhtD 10 μg after each vaccine dose in both the older ($p = 0.0051$ after dose 1, $p = 0.0015$ after dose 2) and young cohorts ($p = 0.0034$ after dose 1, $p = 0.0007$ after dose 2). There was also a statistically significant adjuvant effect after dose 2, indicating a higher antibody response with $AS02_V$ versus alum in both cohorts ($p = 0.0002$ in the older cohort, $p < 0.0001$ in the young cohort). No statistical difference between the PhtD 30 μg and the PhtD 30 μg plus alum groups could be detected in terms of anti-PhtD antibody response in either cohort (data not shown), although the studies were not designed or powered to make this comparison between groups.

In both cohorts, anti-PhtD GMCs were higher 10 months after the second vaccine dose than before vaccination for all PhtD vaccine groups; GMCs remained 2.6- to 4.3-fold higher than pre-vaccination in the young cohort and 2.0- to 4.6-fold higher in the older cohort, with the highest fold increases in the $AS02_V$ groups (Fig. 2).

3.4. Antibody passive transfer in mice

The biological functionality of the anti-PhtD antibodies from participants who received PhtD 30 μg with or without adjuvant was evaluated by passive transfer of serum into naïve recipient mice infected with serotype 3 pneumococci. Fig. 3 details the per group mouse survival following passive transfer of anti-PhtD antibodies from pre-vaccination and post-vaccination sera from vaccinees. The improved survival rates observed in vaccine group PhtD 30 μg plus alum in the young adults, and in PhtD 30 μg plus alum or PhtD 30 μg plus $AS02_V$ in the older cohort, showed that the antibodies generated by the vaccines were functional. For the 3 other groups (plain PhtD 30 μg in both cohorts or PhtD 30 μg plus $AS02_V$ in young adults), the absence of difference may be explained by the higher levels of functionality observed in the prevaccination samples.

3.5. Cell mediated immunity

In both cohorts, memory B cells were boosted by PhtD vaccination with either adjuvant at one month after the second dose (Fig. 4). In older participants, the increase in memory B cells was statistically significantly greater with $AS02_V$ than with alum ($p = 0.0045$), while there was no statistically significant difference between adjuvant groups in the young cohort (Fig. 4).

The frequencies of PhtD-specific CD4 T cells, identified in vitro as expressing two or more cytokines for each of the two intracellular stainings (IFN-γ, IL-2, IL-4, TNF-α; CD40L, GM-CSF, IL-2, TNF-α), were significantly increased by vaccination with PhtD plus $AS02_V$ compared with PhtD plus alum in both cohorts (Table 2). CD40L, IL-2 and TNF-α were the more prevalent expressed markers detected upon in vitro stimulation. No CD8 T cell responses were detected (data not shown).

4. Discussion

Vaccination against pneumococcal protein antigens, such as PhtD, has the potential to offer broad, serotype-independent coverage [15,16]. In the present studies of vaccination with PhtD with or without adjuvantation, solicited local and general reactions were more common in young than in older adults. Reports of grade 3 solicited AEs were infrequent in older adults, with no reports of grade 3 pain or general solicited symptoms. Overall, the reactogenicity profile of $AS02_V$ was acceptable and similar to that reported previously with the $AS02_V$ adjuvant system in healthy adults vaccinated with hepatitis B or HIV antigens [24,37].

A humoral immune response, as measured by anti-PhtD IgG antibody concentration, was demonstrated to the pneumococcal protein PhtD in the older cohort, although this was consistently lower than in the young cohort. The greatest increases were observed in the groups administered adjuvanted PhtD. In particular, use of $AS02_V$ was associated with significantly enhanced anti-PhtD IgG responses in comparison with use of alum adjuvant or plain PhtD vaccination, which was consistent with studies of other vaccines using MPL- and QS21-containing adjuvant systems [38]. $AS02_V$ adjuvantation also induced specific memory B cell and CD4 T cell responses in both cohorts and, interestingly, the $AS02_V$ combination induced significantly higher specific memory B cell levels as compared to the alum combination in the older cohort, similar to those obtained in the young cohort, where no difference between adjuvants was observed. This suggests that $AS02_V$ had a more pronounced effect on memory B cells in older adults than alum, which is important in view of the significant age-related changes in the B cell repertoire that contribute to poor IgM memory B cell responses to 23PPV in older persons [39,40]. The induction of

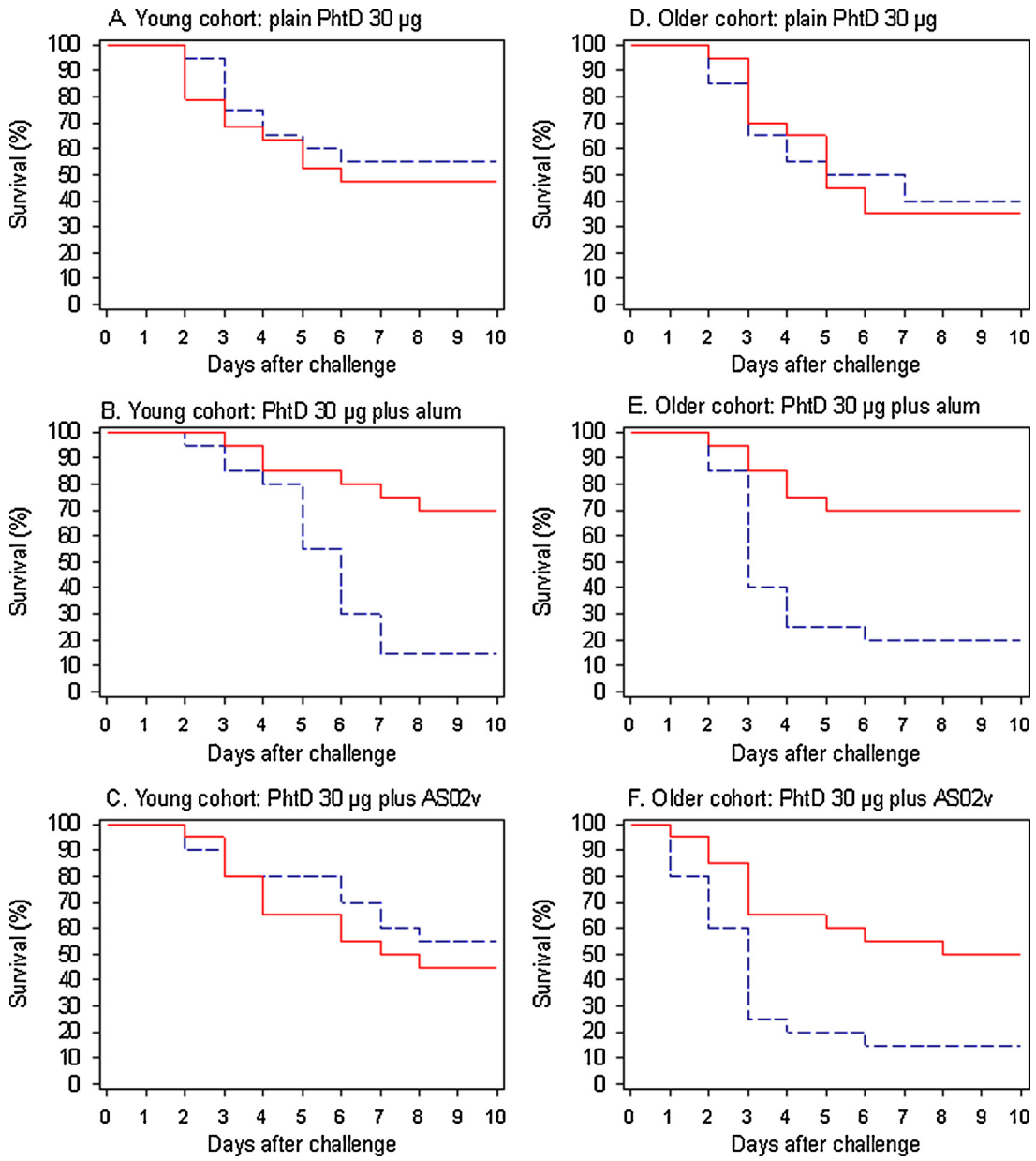

Fig. 3. Mouse survival upon lethal *S. pneumoniae* intranasal challenge following passive transfer of anti-PhtD antibodies. Results shown from experiments of pooled sera from subjects. Blue dotted line: pre-vaccination serum: Red full line: post-vaccination serum. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Frequency of CD4 T cells that produced IFN-γ, IL-2, IL-4, TNF-α, CD40L and GM-CSF, measured in two intracellular cytokine staining assays, *S*1 and *S*2, in young and older participants who received PhtD 30 µg plus alum or PhtD 30 µg plus $AS02_V$ (total vaccinated cohorts). Production of two or more of the markers (*S*1-All doubles and *S*2-All doubles) or any of the markers (*S*1-IFN-γ, *S*1-IL-2, *S*1-IL-4, *S*1-TNF-α, *S*2-CD40L, *S*2-GM-CSF, *S*2-IL-2, *S*2-TNF-α) was evaluated before vaccination (PRE) and one month after the second vaccine dose (M3).

		*S*1-All doubles	*S*1-IFN-γ	*S*1-IL-2	*S*1-IL-4	*S*1-TNF-α	*S*2-All doubles	*S*2-CD40L	*S*2-GM-CSF	*S*2-IL-2	*S*2-TNF-α
Young adult cohort, *n* = 50											
PhtD + alum	PRE	39.1	23.8	46.1	19.3	46.0	136.6	124.7	102.0	66.0	29.3
	M3	649.8	245.4	630.8	50.5	538.3	1229.1	1080.8	429.8	1129.9	662.6
PhtD + $AS02_V$	PRE	95.7	48.4	84.8	49.0	81.0	117.4	65.0	51.2	96.8	67.7
	M3	1885.9	429.9	1846.1	119.6	1782.6	2692.6	2336.5	799.8	2439.2	1895.0
	p value*	0.0011	NS	0.0011	0.0225	0.0004	0.0004	0.0005	0.0023	0.0004	0.0001
Older adult cohort, *n* = 48											
PhtD + alum	PRE	59.8	41.8	33.0	39.6	33.5	78.8	39.3	48.0	57.4	57.7
	M3	200.3	42.8	216.6	20.5	211.8	268.7	92.9	104.4	254.0	210.4
PhtD + $AS02_V$	PRE	50.8	29.0	33.4	30.6	51.3	55.5	26.4	43.3	40.8	27.0
	M3	799.0	81.6	858.0	19.1	833.0	1015.8	365.1	306.0	953.8	842.9
	p value*	0.003	NS	0.0001	NS	0.0001	0.0004	NS	0.0126	0.0002	0.0002

NS = not significant.
* Wilcoxon rank-sum test, PhtD 30 µg plus alum group versus PhtD 30 µg plus $AS02_V$ group at M3.

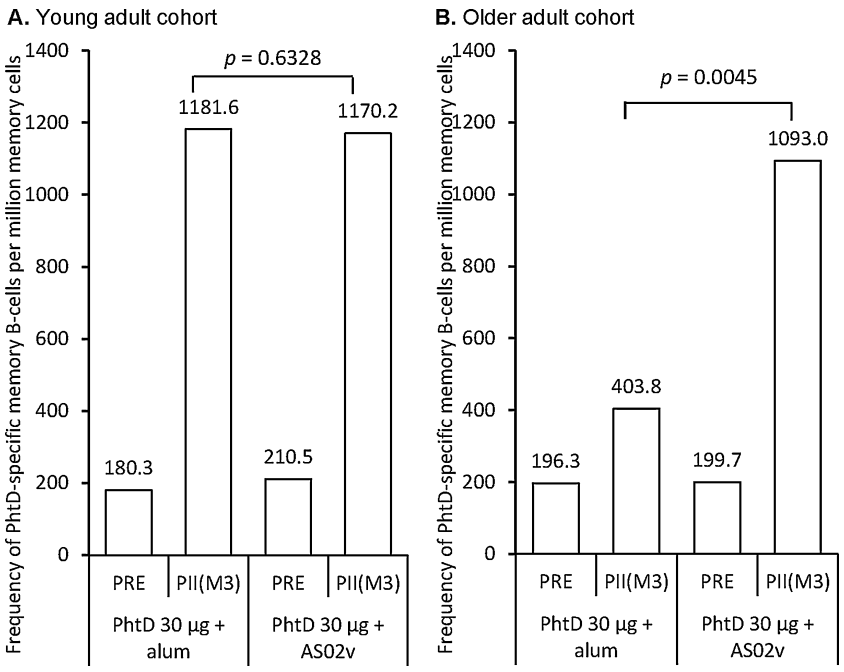

Fig. 4. PhtD-specific memory B cells in the young (A) and older (B) cohorts before vaccination (PRE) and one month after the second vaccine dose (M3) (measured by B cell Elispot assay), expressed as frequency of PhtD-specific Ig-producing B cells per million Ig-producing memory B cells (ATP cohorts for immunogenicity).

CD4 T cell responses in older adults is also significantly higher upon administration with PhtD/AS02$_V$ as compared to PhtD/alum, which is of note since preclinical evidence has suggested that enhancing aged CD4 T cell function leads to improved antibody production [41] and that CD4 T cell interactions with pneumococci are crucial in providing protection in the host [42]. The small sample sizes in each group of our study, however, restrict the ability to make firm conclusions on immune responses induced by PhtD.

A pneumococcal lethal intranasal challenge mouse model was used to test the functionality of vaccine-induced antibodies. The results demonstrated the capacity of anti-PhtD elicited antibodies, regardless of the vaccine, to induce protection against *S. pneumoniae* serotype 3, which is one of the serotypes associated with an increased risk of fatal outcome in older people with invasive pneumococcal disease [43]. This was consistent with other demonstrations of the functionality of human anti-PhtD antibodies in mouse model studies [20,44]. In some groups, the absence of a difference between pre-vaccination and post-vaccination values was likely caused by the relatively high protective potential of baseline sera due to pre-existing antibodies to PhtD or other *S. pneumoniae* antigens resulting from previous pneumococcal exposure, hence precluding the clear demonstration of an increased functionality post-vaccination.

These studies showed that vaccination with the pneumococcal protein PhtD elicits both CD4 T cell and memory B cell responses in adults and that the reduced immune response in older persons compared to young adults can be partially restored with AS02$_V$ adjuvant system. The anti-PhtD antibodies elicited post-vaccination were shown to be functional. No safety concerns were raised and AS02$_V$-adjuvanted PhtD had an acceptable reactogenicity profile. An AS02$_V$-adjuvanted PhtD vaccine may therefore be beneficial in efforts to reduce the burden of pneumonia in the adult population, including older persons.

Disclosure statement

Pneumovax is a trademark of Sanofi Pasteur MSD and *Fluarix* is a trademark of the GlaxoSmithKline group of companies.

Contributors

I.L.-R., G.L.-R. and Y.H. were investigators in these studies and were responsible for the recruitment of subjects, collection and assembly of data, and provided critical input in the protocol, interpretation of results and writing of the manuscript. J.-M.D. and P.V. were involved in all steps of the study from study design to analysis and interpretation of results; P.H. contributed to the design of the study and the interpretation of the results. I.H. and P.M. were responsible for the design, testing and interpretation of humoral response data, and the cellular immune response assessments, respectively. P.V.B. was responsible for the design, execution and interpretation of statistical analyses. V.V. and J.T.P. supervised the design of the study, analysis and interpretation of results. All authors have critically reviewed the manuscript drafts and approved the final article.

Conflict of interest

The study was supported by GlaxoSmithKline (GSK) Biologicals SA. J.-M.D., V.V., I.H., P.M., P.H., and P.V.B. are employees of GSK; J.-M.D., P.M., VV and I.H. owns GSK stock and stock options. J.P. and P.V. were employees of GSK during study conduct. I.L-R, G.L-R, and Y.H. received funding from GSK Biologicals via their institute to cover study costs. I.L-R received fees from GSK Biologicals and Sanofi Pasteur for lectures on vaccine-related topics and received registration and travel expenses from GSK Biologicals to attend vaccine-related conferences G.L-R. received payments from GSK for lectures on HPV vaccines and vaccines in general, for consultancy on influenza vaccines and adjuvants, from Novartis Vaccine and Diagnostics and Immune Targeting Systems (UK) for consultancy on influenza vaccines, and from Baxter Vaccines for lectures on influenza vaccines. Y.H. was involved as investigator in other GSK clinical trials. P.V. received payments from Genticel for consultancy on HPV therapeutic vaccines and from Wittycell for consultancy on adjuvants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.10.052.

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