Impact of adjuvants on CD4+ T cell and B cell responses to a protein antigen vaccine: Results from a phase II, randomized, multicenter trial

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Immunogenicity and safety of different adjuvants combined with a model antigen (HBsAg) were compared. Healthy HBV-naïve adults were randomized to receive HBs adjuvanted with alum or Adjuvant Systems AS01B, AS01E, AS03A or AS04 at Days 0 and 30. Different frequencies of HBs-specific CD4+ T cells 14 days post dose 2 but similar polyfunctionality profiles were induced by the different adjuvants with frequencies significantly higher in the AS01B and AS01E groups than in the other groups. Antibody concentrations 30 days post-dose 2 were significantly higher in AS01B, AS01E and AS03A than in other groups. Limited correlations were observed between HBs-specific CD4+ T cell and antibody responses. Injection site pain was the most common solicited local symptom and was more frequent in AS groups than in alum group. Different adjuvants formulated with the same antigen induced different adaptive immune responses and reactogenicity patterns in healthy naïve adults.

The results summary for this study (GSK study number 112115 – NCT# NCT00805389) is available on the GSK Clinical Study Register and can be accessed at www.gsk-clinicalstudyregister.com.

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1. Introduction

Adjuvants are included in vaccines with the aim of accelerating, prolonging or enhancing the intrinsic immunogenicity of antigens [1]. Aluminum salts were first used as adjuvants in the 1920’s [2] and are still widely utilized in human vaccines. However, aluminum-adjuvanted vaccines are of more limited use when strong T cell responses are required to protect against complex pathogens, chronic infections, or in populations such as the elderly or immunocompromised [5–8]. These limitations have led to the development of variety of new adjuvants based on oil-in-water (o/w) emulsions, saponins and Toll-like receptor agonists. These substances, used alone or in combination, are essential...
components of currently licensed vaccines and candidate vaccines under development [9].

AS01 is an Adjuvant System family containing the TLR4 agonist 3-O-desacyl-4′-monophosphoryl lipid A (MPL) and QS-21 (Quillaja saponaria Molina, fraction 21) formulated with liposomes. It has been developed to potentiate T cell responses against challenging pathogens where classical approaches have proven less effective [10,11]. This is exemplified by the RTS,S/AS01 candidate vaccine which targets Plasmodium falciparum and has been shown to elicit 31% and 50% protective efficacy against clinical malaria in infants and children, respectively [12,13], and by the candidate subunit glycoprotein E varicella zoster HZ/su vaccine inducing >95% protection in older adults [14]. AS03α, an Adjuvant System containing α-Tocopherol and squalene in an o/w emulsion promotes the rapid production of cross-reactive antibodies and allows for antigen-sparing as demonstrated with a pre-pandemic H5N1 candidate vaccine [15,16] and with the licensed H1N1 influenza vaccine [17]. AS04, an Adjuvant System containing MPL adsorbed on Al salt enhances antibody and T cell responses and is included in the licensed human papillomavirus vaccine HPV-16/18 for prevention of cervical cancer [18,19] and in a hepatitis B virus (HBV) vaccine for use in patients with renal insufficiency [20].

These Adjuvant Systems have been shown to induce enhanced antibody and T cell responses in numerous clinical studies targeting a variety of pathogens and some have been compared in clinical trials (e.g., in combination with the candidate malaria RTS,S antigen and the candidate Herpes Zoster antigen), providing valuable information on differential responses in the respective settings [21]. Herpes Zoster antigen), providing valuable information on differential responses in the respective settings [21].

2.1. Study design and participants

This was an observer-blind, randomized, controlled trial (ClinicalTrials.gov identifier: NCT00805389) conducted at 14 study centers (4 in Belgium and 10 in Germany) from December 2008 to July 2011. The protocol was approved by all institutional Ethics Committees and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from each participant prior to trial participation. Eligible participants were healthy men and women aged 18–45 years. Exclusion criteria were: previous vaccination against HBV; positive for anti-HBs antibodies, anti-HBc antibodies, HBSAg, antibodies against hepatitis C virus and/or HIV; previous administration of MPL or QS-21 (Q. saponaria Molina, fraction 21) [Licensed by GSK from Antigenics Inc., a wholly owned subsidiary of Agensia Inc., a Delaware, USA corporation]; administration of any other investigational or non-registered product (drug or vaccine) within the last 30 days or planned use during the study period; administration or planned administration of a vaccine not foreseen by the study protocol within the last 30 days with the exception of the influenza vaccine which could be administered >21 days preceding or following each primary vaccine dose and >7 days preceding or following the booster dose; chronic administration of immunosuppressants or other immune-modifying drugs within the last six months; administration of immunoglobulins and/or any blood products within the last three months, any confirmed or suspected immunosuppressive or immunodeficient condition, history of allergic disease or reactions likely to be exacerbated by vaccine components; other conditions that the investigator judged may interfere with study findings.

A target of 710 eligible participants (142 per group) were to be randomized (1:1:1:1:1) to receive 20 µg HBSAg adjuvanted with alum or one of four GSK proprietary Adjuvants Systems (AS01β, AS01ε containing half the quantity of MPL and QS-21 as in AS01α, AS03α or AS04) at Days 0 and 30. The vaccine formulations are shown in Fig. 1. Vaccine doses were administered by intramuscular injection into the deltoid muscle of the non-dominant arm. The primary endpoint (HBS-specific T cells) was assessed for all participants. Secondary and exploratory endpoints were evaluated in a sub-cohort of participants (375 planned; 75 per group) (Supplementary Fig. 1). Allocation of participants to the sub-cohort was based on HLA type, determined at screening, in order to allow analyses of HBSAg-derived peptide specific T cell responses (see Supplementary Material).

2.2. Treatment allocation and blinding

Participants were allocated a unique treatment number using a centralized randomization system on internet. The randomization algorithm used a minimization procedure accounting for country of recruitment, pre-selected HLA type and gender. When 375 participants had been allocated to the sub-cohort, HLA typing was stopped and the remaining participants were then randomized only according to gender and country. The study was conducted in an observer-blinded manner. Full blinding could not be done due to the different appearance and preparation of the vaccines. Vaccine preparation and administration were performed by authorized medical personnel who did not participate in any of the clinical evaluations. Study participants and those responsible for the evaluation of study endpoints were unaware of group allocation.

2.3. Immunological evaluation

For analyses described here, blood samples were collected on Days 0, 14, 30, 37, 44 and 60 (Fig. 1). All assays were done at central laboratories (ImmuneHealth, Gosselies, Belgium for cell-mediated immunity assays; GSK Vaccines, Rixensart, Belgium for measurement of anti-HBs antibodies) as described below.

2.3.1. HBS-specific CD4+ and CD8+ T cells

HBS-specific CD4+ and CD8+ T cells were measured using frozen peripheral blood mononuclear cells (PBMCs) by the intracellular cytokine staining assay using adaptations of previously described methods [24, 26,27]. Briefly, PBMCs were stimulated in vitro with a pool of peptides (15-mers overlapping by 11 amino acids and covering the entire HBSAg sequence; at 0.5 µg/mL peptide; Eurogentec S.A.) and medium (negative control) for 2 h in the presence of anti-CD28 (CD28.2) and anti-CD49d (9F10) antibodies (from BD Pharmingen). The cells were then stained with extracellular markers, CD4 V450 (SK3) and CD8 Alexa 700 (4S·B3) all from BD Biosciences; IL-13 (eBiosciences Inc) and CD3 Pacific Orange (UCHT1) from Caltag Medsystems Ltd. Finally, cells were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using Flowjo version 9 software (Tree Star Inc., Ashland, OR, USA).
2.3.2. Anti-HBs antibodies

Anti-HBs antibodies were measured using a commercial chemiluminometric immunoassay (CLIA) (Centaur XP™ anti-HBs assay, Siemens Healthcare, Germany). Seropositivity and seroprotection were defined as anti-HBs concentrations ≥ 6.2 mIU/mL and ≥ 10 mIU/mL, respectively.

2.3.3. HBs-specific memory B cells

Frequencies of HBs-specific memory B cells were quantified by enzyme-linked immunosorbent spot (ELISPOT) assay [28]. Briefly, memory B cells were induced to differentiate into plasma cells following in vitro incubation of PBMC with unmethylated DNA (CpG2006 at 3 μg/mL, Eurogentec, Belgium) for 5 days. In vitro-generated plasma cells were then incubated in culture plates previously coated with 5 μg/mL of recombinant HBsAg or with 1 μg/mL of anti-human immunoglobulin G (IgG) (Goat anti-human AffiniPure, Jackson Laboratories) to enumerate specific antibody or IgG secreting plasma cells, respectively. The antibody/antigen spots formed were detected by a conventional immunoenzymatic procedure, as described previously [28]. Data are expressed as number of HBs-specific IgG-producing memory B cells per million of IgG-producing memory B cells.

2.4. Safety evaluation

Solicited local symptoms of pain, redness and swelling and general symptoms of fatigue, fever (oral temperature ≥ 37.5 °C), gastrointestinal (nausea, vomiting, diarrhea or abdominal pain), headache, malaise and myalgia were recorded for 14 days after each vaccine dose. Unsolicited adverse events (AEs) were recorded for 31 days after each vaccine dose. The intensity of symptoms was graded on a 3-point scale. Grade 3 symptoms were defined as redness or swelling > 50 mm in diameter, oral temperature > 39.5 °C, and, for other symptoms, as preventing normal activity. Solicited local symptoms were considered to be causally related to vaccination. The investigator used clinical judgement to determine whether there was a reasonable possibility that the other solicited or unsolicited AE might have been caused by the study vaccine. The occurrence of serious adverse events (SAEs) and AEs of special interest including potential immune-mediated diseases (pIMDs) were reported throughout the entire study period. pIMDs were followed due to the concern of acquiring a vaccine-induced disease of possible autoimmune etiology in susceptible individuals after vaccination with a product containing an adjuvant [29].

Fig. 1. Composition of each vaccine formulation and schedule of assessments to Day 60. The upper part of the figure shows the composition of each vaccine formulation. The study schematic in the lower part of the figure shows the times of vaccine administration for each group (Days 0 and 30) and the timing of collection of blood samples for the immunological endpoints described in the current analysis to Day 60. *also contains squalene in an oil-in-water emulsion. †Al(OH)₃ for Alum group and AlPO₄ for AS04 group.

2.5. Objectives and endpoints

The pre-specified primary objective was to compare the frequency of HBs-specific CD4⁺ T cells expressing at least two immune markers among, CD40L, IL-2, TNF-α, IFN-γ, IL-13 and IL-17, between groups using PBMCs at Day 44. Secondary objectives reported here include between-group comparison of anti-HBs antibody concentrations at Day 60, descriptive summaries of the kinetics of HBs-specific T cell, antibody and memory B cell responses up to Day 60, relationships between adaptive responses, and reactogenicity and safety up to Day 60. *also contains squalene in an oil-in-water emulsion. †Al(OH)₃ for Alum group and AlPO₄ for AS04 group.

Post hoc analyses on the frequency of HBs-specific CD4⁺ T cells expressing at least CD40L and, on the polyfunctionality of CD4⁺ T cells, in those participants who were deemed to be CD4⁺ T cell responders, were conducted (see statistical analysis section). Post hoc analyses also included the between-group comparison of anti-HBs antibody concentrations at Day 44.

Innate immune response analysis, as well as results on adaptive immune response quality (including persistence and boostability) will be reported elsewhere.
2.6. Statistical analysis

In previous clinical trials, AS01 induced a potent cellular response regardless of the antigen or the population [11]. Therefore, the higher dose AS01 group (AS01A) was selected as the primary comparator and sample size was estimated on the basis of comparisons between other adjuvant groups and this group. Assuming a within group standard deviation (SD) of 1.144 on the log_{10} scale, it was estimated that 135 evaluable participants per group (total of 675) would be required to show at least a 3-fold difference between the AS01A group and any of the other groups in terms of the frequency of HBs-specific CD4^+ T cells at Day 44 using Dunnett’s method with a power of at least 88% and a global type I error of 5%. It was assumed that approximately 5% of participants would be excluded from the per protocol analysis, therefore, the target enrolment was 710 participants.

Immunogenicity analyses were based on the according to protocol (ATP) cohort for assessment of adaptive immunogenicity, which included 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 data concerning immunogenicity endpoint measurements were available. The geometric mean of T and B cells’ frequency (GMFs) and the geometric mean of the antibodies’ concentration (GMCs) were calculated. Comparisons of GMFs for HBs-specific CD4^+ T cells and GMCs for anti-HBs antibodies between the AS01A group and other groups used Dunnett’s method via a one-way analysis of variance model. Geometric mean ratios and associated adjusted 95% confidence intervals were calculated. Additional pairwise comparisons between groups used Bonferroni’s adjustment method to control the overall type I error. To evaluate relationships between adaptive immune responses at Days 30, 44 and 60, scatter plots were constructed and post hoc Pearson’s correlation coefficients were calculated. In the primary analysis, data were expressed as number of HBs-specific CD4^+ T cells expressing at least two immune markers per million CD4^+ T cells after subtracting background values. The primary analysis showed background noise at baseline characterized predominately by combinations of cytokines excluding CD40L. Since previous studies have shown that responder cells can be characterized by their expression of CD40L [30–32], post hoc analyses on the frequencies of HBs-specific CD4^+ T cells expressing at least CD40L at Day 44 were performed which allowed reduction of the background noise due to non-specific CD4^+ T cell responses.

For all participants in the ATP cohort, we compared the frequency of CD4^+ T cells expressing at least CD40L at Day 14 and Day 44 between groups using the same methods as those used for the analysis of the pre-specified endpoints (no adjustment for multiple time points performed). For responders, defined as participants with a frequency of CD4^+ T cells expressing at least CD40L above the pre-vaccination threshold (= 95th percentile of the frequency calculated on pooled groups at pre-vaccination), we also summarized the frequencies of HBs-specific CD4^+ T cells expressing any combination of CD40L and the three other markers (IL-2, TNF-α and IFN-γ) at Day 44 using descriptive statistics (bar charts and pie charts). We excluded IL-13 and IL-17 from this analysis of polyfunctionality since post hoc exploratory analyses showed that there was no difference in the frequency of CD4^+ T cells expressing IL-13 and IL-17 between pre- and post-vaccination (Supplementary Fig. 2).

Finally, we calculated a polyfunctionality index [33] for each responder to enumerate the proportion of cells expressing different numbers of immune markers as a single value:

\[
\text{Polyfunctionality index} = F_1 + F_2 + F_3 + F_4
\]

where \( F_1 \) represent the frequencies (%) of cells producing CD40L alone and \( F_2, F_3 \) and \( F_4 \) represent the frequencies (%) of cells expressing CD40L in combination with 1, 2 or 3 markers, respectively, with \( F_1 + F_2 + F_3 + F_4 = 100\% \). Polyfunctionality indices were summarized for each group using descriptive statistics.

Descriptive summaries of demographic and safety data were based on the total vaccinated cohort, which included all participants for whom at least one vaccine administration was documented.

Statistical analyses were done using SAS version 9.2 (SAS Institute, Cary, NC, USA) and ProcStatXact 8.1 (Cytel Inc., Cambridge, MA, USA).

3. Results

3.1. Study population

The composition of each vaccine as well as the schedule for administration and assessments are given in Fig. 1. A total of 713 participants were randomized and received at least one vaccine dose and 691 participants completed the study to Day 60, with the number of participants who withdrew from the study being similar among groups (Supplementary Fig. 1). A total of 623 participants received two vaccine doses. Temporary suspension of dosing was undertaken due to two cases of autoimmune hepatitis in a separate study evaluating an AS03A-adjuvanted vaccine. The two cases were found to be in existence before vaccination and thus unrelated. Dosing was resumed after 4 weeks, but for 74 participants the second dose was not administered because it would have been out of schedule. These participants were not included in the ATP cohort (for full details see Supplementary Material) and were evenly distributed among groups. The ATP cohort for assessment of adaptive immunogenicity included 599 (84%) participants. The major reason for exclusion from the ATP cohort was that vaccine doses were not administered according to the planned schedule (80 [11%] participants, of which 74 received only one vaccine dose due to the temporary suspension of dosing). A total of 373 participants were enrolled in the sub-cohort for evaluation of antibody and memory B cell responses, and of these, 293 (79%) were included in the ATP cohort. The groups were well matched in terms of age, race and gender (Table 1).

3.2. Impact of adjuvants on the magnitude of CD4^+ T cell responses

The magnitude of the HBs-specific CD4^+ T cell responses was measured as the frequency of cells expressing at least two markers among six, in response to ex vivo stimulation with peptides spanning the HBs antigen, as done previously [24]. An additional post hoc analysis based on the frequency of cells expressing at least CD40L upon antigen stimulation was also conducted. In the AS01A, AS01B and AS03A groups, the GMFs of HBs-specific CD4^+ T cells initially peaked 14 days after the first vaccine dose and then declined over the ensuing two weeks. Levels increased again after the second vaccine dose and a second higher peak was observed at Day 44, both for cells expressing at least CD40L and for cells expressing at least two markers. In the alum group, GMFs of CD4^+ T cells increased more steadily over the observation period (Fig. 2A and Supplementary Fig. 3).

The AS01A- and AS01B-based formulations induced the highest GMFs of HBs-specific CD4^+ T cells and no statistically significant differences were observed between these two groups. For cells expressing at least CD40L, GMFs were statistically higher for both the AS01A and AS01B groups compared with the AS03A group at Day 44 (1.83 [1.28, 2.62]- and 1.69 [1.18, 2.43]-fold higher, respectively), which in turn was higher than the AS04 group (2.48 [1.74, 3.53]-fold) and the alum group (3.23 [2.26, 4.61]-fold). No statistically significant difference in the frequencies of CD4^+ T cells was observed between the AS04 and alum groups. Similar results were observed when CD4^+ T cell responses were measured as GMFs of cells expressing at least two of six markers (Supplementary Fig. 3 and Supplementary Table 1). For cells expressing at least CD40L, no differences could be observed at Day 14, with the GMF of AS01A 0.86 (0.63, 1.16)-fold lower than AS03A, and the GMF of AS01B 0.92 (0.66, 1.29)-fold lower than AS03A at this earlier time point (Supplementary Table 1).
No HBs-specific CD8$^+$ T cells were detected in any of the groups (data not shown) as was the case in other studies using recombinant protein antigens [24,34].

### 3.3. Impact of adjuvants on the magnitude of B cell responses and antibodies

SIGNIFICANT differences were observed in the magnitude and kinetics of antibody and memory B cell responses between the groups. In the AS01E, AS01E and AS03A groups, anti-HBs antibody titers increased after each of the two vaccine doses (Fig. 2B) and reached statistically significant higher GMCs at Day 44 and Day 60 compared with the alum group, which in turn was statistically significantly higher than the AS04 group. A statistically significant difference was observed between GMCs in the AS01E group vs. the AS03A group at Day 44 (2.17 [1.13, 4.18]-fold higher) but not at Day 60 (Supplementary Table 2). No statistically significant differences in anti-HBs GMCs were observed between the AS01E and AS03A groups at Day 44 or Day 60 (Supplementary Table 2). According to the measurements performed at Day 44 and

![Graphs](image)

**Fig. 2.** Effect of different adjuvants on the kinetics of the adaptive immune responses for the ATP cohort. Plots show (A) the frequency of HBs-specific CD4$^+$ T cells expressing at least CD40L, (B) the concentration of anti-HBs antibodies; and (C) the frequency of HBs-specific memory B cells (on the sub-cohort for antibody and memory B cell responses) for participants receiving vaccine doses on Day 0 and Day 30 consisting of HBsAg 20 μg/dose, adjuvanted with alum (black), AS04 (red), AS03A (green), AS01E (light blue) and AS01A (dark blue). Geometric mean values (geomean) and 95% confidence intervals are shown.

Table 1

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Characteristic</th>
<th>Group</th>
<th>Number of participants</th>
<th>Age (years) at first vaccination, mean (SD)</th>
<th>Female, n (%)</th>
<th>Race, n (%)</th>
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ATP, according-to-protocol cohort for adaptive immunogenicity; n (%), number (percentage) of participants in a given category; SD, standard deviation; TVC, total vaccinated cohort.
Day 60, the maximum antibody titer was also reached earlier in the AS01 and AS03A groups (observed at Day 44 for 87.5% and 80.7% of participants, respectively) as compared with the AS04 group (53%) and the alum group (26%) (Supplementary Table 3).

At Day 60, antibodies levels of ≥ 10 mIU/mL were reached for 100% of participants in the AS01 group, AS01E and AS03A groups, 93.2% of participants in the AS04 group and 58.2% of participants in the alum group.

As observed for antibodies, the frequencies of HBs-specific memory B cell responses increased after each vaccine dose in the AS01g, AS01E, AS03A groups and after the second vaccine dose in the AS04 and alum groups, with no or low increase observed post dose 1. In the four Adjuvant Systems groups, the B cell responses tended to peak between Days 30 and 44 (Fig. 2C). In the alum group, the B cell response tended to take longer to reach the maximum observed value compared to the other groups. Similar to antibodies, observed GMFs of HBs-specific memory B cells were higher in the AS01g, AS01E, AS03A groups than in the AS04 and alum groups (GMFs for HBs-specific memory B cells at Day 44 were 3729, 4042, 1436, 28 and 14 per 10⁶ cells, respectively, but no statistical comparisons were performed) (Supplementary Table 4).

3.4. Relationship between T cell and antibody responses

We explored whether antigen-specific T cells measured in the blood, monitored via expression of CD40L or cytokines upon restimulation were correlated to antibody levels. Correlations between the frequencies of HBs-specific CD4⁺ T cells and anti-HBs antibody titers were generally low (Fig. 3). At Day 44 and 60, the highest correlations were observed in the AS03A group (Pearson’s correlation coefficient, R = 0.6 and 0.5 respectively). Observed correlations were lower in the AS01g group (R ≤ 0.4) and were even very low in the AS01E group (R ≤ 0.14), as shown by the more vertical position of the predictive ellipse for this group. Correlations in the AS04 and alum groups should be considered with caution considering the low numbers of responders for the antibody and CD4⁺ T cell responses.

3.5. Polyfunctionality of CD4⁺ T cell responses to adjuvants

Adjuvants could potentially induce qualitative, in addition to quantitative, differences in the CD4⁺ T cell responses [1], because they differentially activate innate immunity.

As a first approach to test this hypothesis, the polyfunctionality of HBs-specific CD4⁺ T cells was analyzed. The analysis was focused on CD4⁺ T cells producing at least CD40L and was restricted to participants showing a response to the formulations. As shown in Fig. 4, the proposed intracellular cytokine staining assay showed that the HBs-specific CD4⁺ T cells induced by the five vaccine formulations had similar patterns of polyfunctionality. Similar percentages of cells producing 1, 2 or 3 markers among CD40L⁺ cells were detected in the different groups (Fig. 4A). The predominant populations were cells producing CD40L only or in combination with either IL-2, or IL-2 and TNF-α. Low frequencies of cells producing IFN-γ were only detected in the AS01g and AS01E groups (Fig. 4A and B). In order to quantify the polyfunctionality of HBs-specific CD4⁺ T cells, an index was calculated in which polyfunctional cells have a higher weight than pauci-functional cells [33]. As shown in Fig. 4C, the polyfunctionality indexes of HBs-specific CD4⁺ T cells were similar in the five groups.

3.6. Safety and reactogenicity

The differences in immunogenicity of the five vaccine formulations were associated with differences in their reactogenicity profiles, with an apparent lower reactogenicity in the alum group than in the four Adjuvant System groups (Fig. 5 and Supplementary Table 5). A tendency towards a higher frequency of solicited symptoms was observed for the AS01g-adjuvanted vaccine as compared to the other adjuvanted formulations. In all groups, the symptoms were transient with the highest frequency occurring within the first two days after dosing and prevalence declined rapidly thereafter (Supplementary Figs. 4 and 5). Only a small proportion of participants had symptoms beyond Day 6.

A larger proportion of participants in the Adjuvant System vaccine groups had pain at the injection site, local redness or local swelling compared to the alum group (Fig. 5 and Supplementary Table 5). Grade 3 local symptoms were most common in the AS01g group whereas no participants in the alum group experienced grade 3 local symptoms. The most frequently solicited general symptoms were fatigue, headache and myalgia and these symptoms were more frequent in the AS01E group than other groups (Fig. 5 and Supplementary Table 5). Grade 3 general symptoms occurred at low frequencies and were generally comparable across groups.

In general, reactogenicity (solicited local and general symptoms) did not increase after the second vaccine dose, except in the AS01g group, for which systemic, but not local, reactogenicity tended to be higher after dose 2 (Fig. 5 and Supplementary Table 5).

Unsolicited AEs and SAEs up to Day 60 are described in the Supplementary Material and Supplementary Table 6. The prevalence and type of unsolicited AEs was similar in each group (~50% of participants). None of the reported AEs leading to withdrawal (3 participants) or SAEs (15 events for 12 participants) were considered to be related to vaccination by the investigator.

4. Discussion

Here, we describe cellular and humoral immune responses, as well as reactogenicity and safety parameters, following vaccination with two doses of study vaccines up to Day 60. The current direct comparison should provide data assisting adjuvant selection decisions in future vaccine development and establishes a framework in which the value of new adjuvants can be evaluated.

Several conclusions can be drawn. First, the different HBsAg vaccine formulations induced markedly different adaptive immune reactions, which was expected given the current clinical experience with the adjuvant systems. In addition, the use of Adjuvant Systems allows a faster onset of the immune response as compared to alum, with the highest response obtained after two weeks post immunization. However, despite being quantitatively different, CD4⁺ T cells induced by the different vaccine formulations displayed comparable functionality profiles, dominated by expression of IL-2 and CD40L. Second, no obvious correlations between antibody titers and CD4⁺ T cell frequencies were observed although there was a trend towards less variability in antibody titers with increasing CD4⁺ T cell frequencies. Third, reactogenicity profiles differed between the different formulations with the interesting observation that for the HBsAg/AS01g vaccine, reactogenicity after the second dose seemed to be higher than after the first dose.

4.1. Cellular immune responses

In terms of CD4⁺ T cell and B cell responses, the highest frequencies were observed following vaccination with the AS01g or AS01E adjuvanted vaccines. Immune responses appeared to show a dichotomy with AS01g, AS01E, AS01E and AS03A, inducing relatively stronger responses on one hand, and AS04 and alum inducing relatively weaker responses on the other hand. Immune responses were already detected after the first dose for the AS01 and AS03A adjuvanted vaccines with no clear differentiation between AS01 and AS03A groups in this population of naïve adults. Responses rose sharply after two vaccine doses and at subsequent sampling times. It is of interest that the AS04-adjuvanted vaccine induced lower frequencies of CD4⁺ T cells as compared to AS01. AS04 also contains MPL but adsorbed on an aluminum salt in an aqueous solution rather than combined with QS-21 in a liposome formulation as in AS01. These results are consistent with mouse studies indicating that the MPL and QS-21 components of AS01 act synergistically rather than additively, to stimulate induction of CD4⁺ T cell responses [35]. This is not seen when MPL...
is adsorbed on an aluminum salt [35,36]. These observations illustrate the potential added value of combining different active components in an Adjuvant System to support the increased induction of CD4+ T cell and B cell responses. Induction of robust CD4+ T cell responses was not limited to the AS01 formulations. The AS03A-based formulation also induced high frequencies of HBs-specific CD4+ T cells, albeit lower than in the AS01 groups. Formulation with AS03 also boosted antibody responses. Although the vaccine formulations induced quantitatively different T and B cell responses, the functional profiles of HBsAg-specific CD4+ T cells were strikingly similar in the five study groups, as measured by the intracellular cytokine staining assay. The dominant functional markers produced were CD40L, IL-2 and, to a lesser extent TNF-α, whereas relatively small proportions of antigen-specific cells produced IFN-γ. These observations are surprising given the different nature of the adjuvants included in the formulations and their reported effect on T cell differentiation in animal models [36–38]. Thereby, the different effect of adjuvants on innate immunity may not directly translate into a different quality of T cell responses in humans but rather result in an
Fig. 4. Polyfunctionality of the CD4⁺ T cell responses induced by different adjuvants for participants defined as "responders" in the ATP cohort. Pie charts (A) and bar graphs (B) of cytokine co-expression profiles of HBsAg-specific CD4⁺ CD40L⁺ T cells at 2 weeks post-dose II (Day 44) in the five study groups for participants in the ATP cohort for adaptive immunogenicity who were defined as "responders" (defined as participants with frequency of CD4⁺ T cells expressing at least CD40L above threshold = 95th percentile of the frequency calculated on pooled groups at pre-vaccination). Results were expressed as the percentage in pie charts (A) or as the absolute cell numbers in bar graph (B) of the total CD4⁺CD40L⁺ T cells expressing 1, 2 or 3 cytokines (IL-2, TNF-α or IFN-γ). Individual results and median of polyfunctionality index (C) calculated on CD4⁺ T cells expressing at least CD40L, alone or with an additional 1, 2, or 3 markers.
Fig. 5. Radar plots of solicited local and general symptoms after each dose for the total vaccinated cohort. Each spoke on the radar plot shows one of the solicited local or general symptoms (clockwise from top: PA, pain; RE, redness; SW, swelling; FE, fever; FA, fatigue; HE, headache; MY, myalgia; MA, malaise; GI, gastrointestinal). The outer end of each spoke represents 100% of participants, and the inner end of each spoke represents 0% of participants. The green dot-dashed line shows the percentage of participants with any grade of the symptom, the blue solid line shows the percentage of participants with grade 1 of the symptom and the red dashed line shows the percentage of participants with grade 2 of the symptom.
increase in the magnitude of the response. A limitation is the low number of parameters measured. A more extensive analysis of secreted cytokines or gene expression profiles may reveal differences and this analysis is ongoing. Indeed, distinct transcriptional signatures were detected in murine T cells that appeared to be similar by flow cytometric analysis [39]. In addition, only peripheral T cells were measured, which may not reflect what is ongoing in the lymph node or germinal centers.

No antigen-specific CD8⁺ T cell responses were detected in any of the adjuvant groups, confirming earlier data from clinical studies. In preclinical studies, however, CD8⁺ T cell responses could be detected [11,40–42]. In mice, spleen cells [40] and PBMCs [41] were examined and in rhesus macaques [41,42] PBMCs were re-stimulated with peptides in vitro before cytotoxic T lymphocyte (CTL) analyses. Moreover, in humans, CTL analyses have been demonstrated with adjuvanted HBsAg vaccination after in vitro expansion of the cells [43]. Therefore, comparisons need to be made with great care and with methodological differences in mind.

### 4.2. Antibody responses

At Day 60, one month after the second vaccine dose, all participants in the AS01b, AS01e, and AS03x groups had reached anti-HBs levels of ≥10 mIU/mL, compared with 93% in the AS04 group and 58% in the alum group. Significantly higher antibody responses and numerically higher rates of antibody levels ≥10 mIU/mL were detected in the AS04 group as compared to the alum group, despite the induction of similar frequencies of HBsAg-specific CD4⁺ T cell and memory B cells. This capacity of AS04 to boost antibody responses is further illustrated by its capacity to improve antibody responses to HBsAg in patients with end stage renal disease [44,45] and to HPV-16/18 antigens in adolescents and adults [46]. The relatively low rates of antibody levels ≥10 mIU/mL observed in the alum group are consistent with the need of three doses to achieve seroprotection in most HBsAg-naïve individuals. CD4⁺ T cell frequencies and antibody titers correlated poorly up to Day 60 despite displaying similar hierarchies between the vaccine formulations. Further analyses are needed to evaluate whether the CD4⁺ T cell frequencies and qualities can predict the persistence of the humoral response beyond Day 60.

### 4.3. Reactogenicity

The differential magnitudes of T and B cell responses to the vaccine formulations were associated with differences in reactogenicity. Pain was the most common solicited AE reported by participants receiving AS-adjuvanted vaccines and this occurred at higher frequency and intensity as compared to alum. An increase in local reactogenicity may reflect the local activation of the innate immune system by Adjuvant Systems, as described in mice and in vitro on human cells [36–38]. Local reactogenicity was more pronounced in the AS01b group compared to the AS01e group (containing half the amount of MPL and QS-21). Interestingly, systemic reactogenicity was higher after the second vaccine dose as compared to the first for the AS01b group, while local reactogenicity remained unchanged. This was not observed for any of the other groups. It will be of interest to better understand the mechanism behind this dose-dependency of systemic reactogenicity. The AS01e and AS01x groups did not differ significantly in terms of CD4⁺ T cell and antibody responses, indicating that comparable immune responses can be induced with reduced reactogenicity, at least in the current study population, i.e. HBsAg-naive, younger adults.

### 5. Conclusions

Although the present study allows for an objective and informative comparison of adjuvants, the results cannot simply be extrapolated to other populations and antigen/adjuvant combinations. Population differences in terms of age (e.g., children vs. elderly), immune status (immunocompromised vs. healthy), physiological status (pregnancy), factors pertaining to the host or ethnic origin (e.g., HLA haplotype or other genetic host determinants) and/or pre-existing immunity (i.e., naïve vs. primed) have to be taken into account when selecting adjuvants for any vaccine candidate.

In conclusion, this study shows that different adjuvants have a profound impact on the kinetics and the magnitude of T and B cell responses to a given protein vaccine in humans. Further analyses from this study should determine the impact of adjuvants on the quality of these responses and on their relationship with innate immune responses and with reactogenicity. Taken together, these analyses will provide essential information for the selection of adjuvants in the development of efficacious and safe vaccines.

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### Conflict of interest

AD, FT, IC, LF, MJ, PM, PVB, RV and WB, are employees of the GSK group of companies. AD, FT, PM, PVB and RV own GSK restricted shares. AH has no conflict of interest to disclose. AM, EH and JL's institution received payment from the GSK group of companies for the performance of the study as well as consulting fee.

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MV and NG were employees of the GSK group of companies and own GSK stocks or stock options. NG has granted issued patents for AS04 and AS01.

PVD's institution received grants and non-financial support from the GSK group of companies, during the conduct of the study as well as payment or reimbursement for travel costs and accommodation for lectures including service on speaker bureaus from several vaccine companies, including GSK Vaccines, Merck, Novartis, Gentecel, SP-MSD, Pfizer, Sanofi-Pasteur.

TS received honoraria from the GSK group of companies as a consultant, member of advisory boards, lecturing and conducting clinical trials. YH's and WJ's respective institutions received payment from the GSK group of companies for performing the study.

### Author contributions

AD, AM, GL, MV, NG, PVB, WB participated in the conception or design. AH, AM, GL, JL, PK, PVD, YH participated to the planning of the study on sites. The investigators or co-investigators, AH, EH, FC, GL, JG, ME, PK, PVD, TS and WJ, together with the following other authors: AM, IC, LF, MJ, PM, PB and WB, participated to the collection and/or assembling of the data. Performing or supervising the analysis was done by AH, AM, FT, IC, JC, LF, MJ, MV, PM, PVB, TS, WB. IC and LF provided with statistical expertise. AD, AM, GL, LF, MV, NG, PVB, RV, TS and WB...
participated to the interpretation of results with the help of AH, EH, FC, FT, IC, JC, JL, MJ, PM and PVD. Drafts were developed by a professional publication writer according to the recommendations, documentations and outline provided by the authors. GL, AD, AM and RV also took an active role in the writing of the manuscript. All authors had full access to the data, reviewed and approved the present manuscript for submission and agree to be accountable for all aspects of the work.

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Appendix A Supplementary data

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