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Vaccine Adjuvant Systems containing monophosphoryl lipid A and QS-21 induce strong humoral and cellular immune responses against hepatitis B surface antigen which persist for at least 4 years after vaccination



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ABSTRACT

Background: Recombinant hepatitis B surface antigen (HBsAg) was used as a model antigen to evaluate persistence of cellular and humoral immune responses when formulated with three different Adjuvant Systems containing 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and QS-21, in an oil-in-water emulsion (AS02_B and AS02_V), or with liposomes (AS01_B).

Methods: This is an open, 4-year follow-up of a previous randomised, double-blind study. Healthy subjects aged 18–40 years received three vaccine doses on a month 0, 1, 10 schedule and were initially followed for 18 months. A total of 93 subjects (AS02_B: n = 30; AS02_V: n = 28; AS01_B: n = 35) were enrolled in this follow-up and had an additional blood sample taken at Year 4 (NCT02153320). The primary endpoint was the frequency of HBsAg-specific CD4⁺ and CD8⁺ T-cells expressing cytokines upon short-term *in vitro* stimulation of peripheral blood mononuclear cells with HBsAg-derived peptides. Secondary endpoints were anti-HBs antibody titres and frequency of HBsAg-specific memory B-cells.

Results: A strong and persistent specific CD4⁺ T-cell response was observed at Year 4 in all groups. HBsAg-specific CD4⁺ T-cells expressed mainly CD40L and IL-2, and to a lesser extent TNF- α and IFN- γ . HBsAg-specific CD8⁺ T-cells were not detected in any group. A high, persistent HBsAg-specific humoral immune response was observed in all groups, with all subjects seroprotected (antibody titre \geq 10 mIU/mL) at Year 4. The geometric mean antibody titre at Year 4 was above 100,000 mIU/mL in all groups. A strong memory B-cell response was observed post-dose 2, which tended to increase post-dose 3 and persisted at Year 4 in all groups.

Conclusion: The MPL/QS-21/HBsAg vaccine formulations induced persistent immune responses up to 4 years after first vaccination. These Adjuvant Systems offer potential for combination with recombinant, synthetic or highly purified subunit vaccines, particularly for vaccination against challenging diseases, or in specific populations, although additional studies are needed.

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Abbreviations: AS, adjuvant system; DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunosorbent spot; GMT, geometric mean antibody titre; HBsAg, hepatitis B surface antigen; HIV, human immunodeficiency virus; ICS, intracellular cytokine staining; IFN γ , interferon gamma; IL2, interleukin 2; MPL, 3-O-desacyl-4'-monophosphoryl lipid A; PBMC, peripheral blood mononuclear cells; QS-21, *Quillaja saponaria* Molina fraction 21; TNF α , tumour necrosis factor alpha.

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1. Introduction

GlaxoSmithKline (GSK) Vaccines have developed a series of Adjuvant Systems (AS) containing two potent immunostimulants, 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) and QS-21 (*Quillaja saponaria* Molina fraction 21; Antigenics Inc, a wholly owned subsidiary of Aenus Inc., Lexington, MA, USA), which in addition to promoting strong and persistent antibody responses, potentiate specific cellular immunity [1]. Such Adjuvant Systems may be useful to enhance potency in vaccines against pathogens that cause complex acute or chronic disease (e.g., human immunodeficiency virus (HIV), *Plasmodium falciparum*, *Mycobacterium tuberculosis*, or herpes zoster in older adults [2,3]), in therapeutic vaccines (e.g., cancer vaccine targeting MAGE-A3 [4]), or to induce immune responses in populations that do not adequately respond to non- or alum-adjuvanted vaccines (e.g., herpes zoster and *Streptococcus pneumoniae* in older adults [5–7]).

Administration of vaccines containing both MPL and QS-21 have resulted in high antigen-specific antibody and CD4⁺ T-cell responses in preclinical and clinical studies [8–18]. The next challenge in Adjuvant System research is to evaluate effects on the magnitude and quality of immune responses over the longer term, particularly with regard to cytokine production by polyfunctional T-cells. To our knowledge, no long-term immunological studies of adjuvanted vaccines have been published.

In the present 4-year follow-up to a previously reported study [8], we evaluated the persistence of immune responses using hepatitis B surface antigen (HBsAg) as a model antigen formulated with three different MPL and QS-21-containing Adjuvant Systems: AS02_B, AS02_V, and AS01_B. The primary objective was to evaluate the persistence of cellular immune responses in terms of HBsAg-specific CD4⁺ and CD8⁺ T-cells. Secondary objectives were to evaluate persistence of anti-HBs antibodies and HBsAg-specific B-cell memory.

2. Methods

2.1. Study design and participants

This was an open, 4-year follow-up of a randomised, double-blind study conducted at two centres in Belgium [8]. No additional vaccines were administered in this follow-up study. There was a single visit at Year 4 at which a blood sample for immunogenicity assays were taken. No further follow-up is planned after this 4-year time point. Samples were collected between September and October 2005. All subjects gave written informed consent for the follow-up evaluation and the study was approved by the local ethics committees according to the Declaration of Helsinki. The study is registered with ClinicalTrials.gov (number NCT02153320).

In the primary vaccination study, healthy male and female subjects were vaccinated on a month 0, 1, 10 schedule with three doses of recombinant HBsAg (20 µg) formulated with the following Adjuvant Systems: AS02_B (100 µg MPL and 100 µg QS-21 in full dose of oil-in-water emulsion), AS02_V (50 µg MPL and 50 µg QS-21 in reduced amount of modified oil-in-water emulsion), AS01_B (50 µg MPL and 50 µg QS-21 in liposomes), or CpG 7909 class B oligonucleotide (500 µg in saline buffer) [8]. Participants were recruited from the 131 subjects who completed the primary study [8], who had been randomised to one of the three groups that received recombinant HBsAg formulated with AS02_B, AS02_V or AS01_B and who consented to follow-up evaluation. Subjects who received HBsAg formulated with CpG were not enrolled in this 4-year follow-up because of their poor cellular immune responses up to Week 78 [8].

A delayed-type hypersensitivity (DTH) skin test involving intradermal injection of 2 µg purified HBsAg was performed at Week 78 of the primary vaccination study, as reported previously [8]. This test was stopped prematurely because of reactogenicity [8], therefore, some subjects enrolled in the 4-year follow-up had received the DTH challenge and some had not.

2.2. Immunological assays

2.2.1. HBsAg-specific CD4⁺ and CD8⁺ T-cells

HBsAg-specific CD4⁺ and CD8⁺ T-cell responses were evaluated at Week 78 (using existing samples collected during the double-blind study) and at Year 4, using Intracellular Cytokine Staining (ICS) as described for the previous study [8]. Briefly, following short-term *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with a pool of peptides covering the entire sequence of the recombinant HBsAg, cells were processed by conventional immunofluorescence and analysed by flow cytometry to measure the frequency of HBsAg-specific CD4⁺ or CD8⁺ T-cells secreting CD40L, interleukin 2 (IL2), tumour necrosis factor alpha (TNFα), and/or interferon gamma (IFNγ) (frequencies expressed per million cells).

2.2.2. Serum anti-HBsAg antibodies

Anti-HBsAg antibody titres were evaluated at Year 4 by AxSYM[®] AUSAB[®] assay (Abbott Laboratories). For the cohort of subjects enrolled in this follow-up, anti-HBsAg antibody titres previously measured at Day 0 and Weeks 6, 46, 48 and 78 are also summarised.

2.2.3. HBsAg-specific memory B-cells

HBsAg-specific B-cell responses were evaluated at Day 0, Weeks 6, 46, 48 and 78 (using existing samples collected during the double-blind study) and at Year 4. Memory B-cells were induced to differentiate into plasma cells following *in vitro* cultivation of PBMC with unmethylated DNA (CpG2006 at 3 µg/mL, Eurogentec, Belgium) for 5 days. *In vitro*-generated antigen-specific plasma cells were enumerated using the enzyme-linked immunosorbent spot (ELISPOT) assay [19]. *In vitro*-generated plasma cells were incubated in culture plates previously coated with 5 µg/mL of antigen or with 100 µL of anti-human IgG at 1 µg/mL (Goat anti-human Affinipure, Jackson Laboratories) in order to enumerate specific antibody or IgG secreting plasma cells, respectively. The antibody/antigen spots formed were detected by a conventional immunoenzymatic procedure. Frequencies of HBsAg-specific memory B-cells are expressed per million IgG-producing memory B-cells.

2.3. Safety

Reactogenicity and safety of the vaccine formulations up to Week 78 were described in the earlier publication [8] and no additional safety evaluation was done at Year 4.

2.4. Statistical methods

The total cohort included all subjects enrolled in this follow-up study. For each specified endpoint, those subjects for whom validated assay results were available were included in the analysis of that endpoint. The number of subjects included in analyses differs by endpoint as validated assay results were not available for all subjects for every endpoint. Data are summarised using descriptive statistics. No statistical comparisons were made.

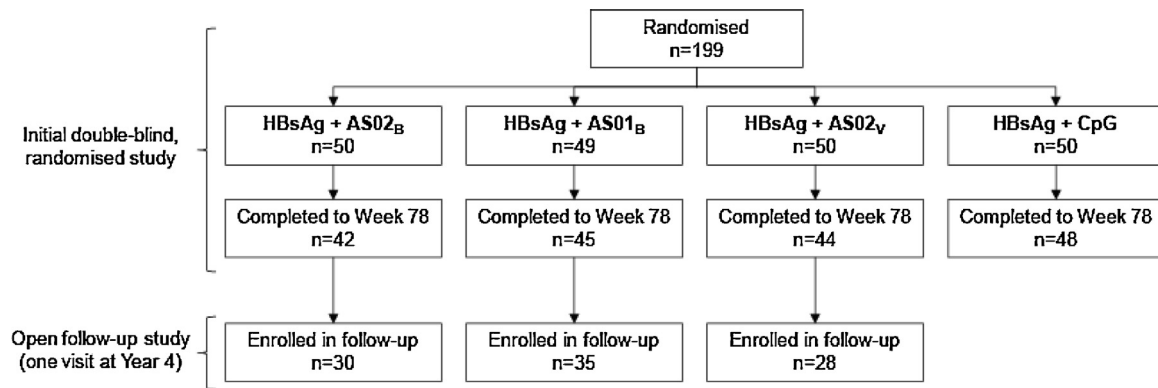


Fig. 1. Flow of participants through the study.

2.4.1. Cell-mediated immune responses

The frequency of HBsAg-specific CD4⁺ and CD8⁺ T-cells expressing at least two markers among CD40L, IL2, TNF α , and IFN γ per million CD4⁺ or CD8⁺ T-cells, respectively, and the frequency of HBsAg-specific memory B-cells per million IgG-producing memory B-cells, are summarised. The cytokine co-expression profile of HBsAg-specific CD4⁺ and CD8⁺ T-cells is also summarised. Log-transformation of the frequency of HBsAg-specific cells was applied by setting all values of 0 equal to 1.

2.4.2. Serum anti-HBsAg antibodies

Seroprotection rates and geometric mean antibody titres (GMTs) with 95% confidence intervals (CIs) were calculated. GMTs were calculated by taking the anti-log of the mean of the log titre transformations. Antibody titres below the cut-off of the assay were given an arbitrary value of half the cut-off for the purpose of GMT calculation. Seroprotection was defined as an anti-HBsAg antibody titre ≥ 10 mIU/mL.

2.4.3. Post-hoc analysis according to delayed-type hypersensitivity DTH challenge

To investigate the impact of the DTH challenge on immune responses at Year 4, exploratory dotplots of immune responses at Week 78 versus Year 4 were produced for the subpopulations of subjects who received the DTH challenge and those who did not.

3. Results

3.1. Study population

A total of 93 subjects were enrolled in this 4-year follow-up (30 in the AS02_B group, 35 in the AS01_B group and 28 in the AS02_V group) (Fig. 1). The DTH challenge at Week 78 was performed on 83 of these subjects (26 in the AS02_B group, 30 in the AS01_B group and 27 in the AS02_V group).

The three groups were comparable with respect to age, with an overall mean age of 28.1 years (range 23–44 years). Overall, 65% of the subjects were male (between 57% and 70% depending on the group) and the population was predominantly white Caucasian (98%).

3.2. HBsAg-specific CD4⁺ and CD8⁺ T-cells

Following *in vitro* stimulation of PBMCs, a strong HBsAg-specific CD4⁺ T-cell response (*i.e.*, cells expressing at least two markers among CD40L, IL2, TNF α and IFN γ) was observed at Week 78 and Year 4 in all three groups (Fig. 2). The median frequencies of

HBsAg-specific CD4⁺ T-cells expressing at least two markers per million CD4⁺ T-cells at Week 78 and Year 4, respectively, were 3400 and 2433 for AS02_B, 2227 and 2189 for AS01_B, and 1338 and 1093 for AS02_V.

The polyfunctional HBsAg-specific CD4⁺ T-cell response persisted between Week 78 and Year 4 (Fig. 3). At Year 4, HBsAg-specific CD4⁺ T-cells expressed mainly CD40L and IL2, and to a lesser extent TNF α and IFN γ , with a similar profile in each group (Fig. 4).

No significant levels of HBsAg-specific CD8⁺ T-cells could be detected in any of the groups at Week 78 or at Year 4 following *in vitro* stimulation of PBMCs with the HBsAg peptide pool (median <40 HBsAg-specific CD8⁺ T-cells per million CD8⁺ T-cells per group).

3.3. Serum anti-HBsAg antibodies

In all three groups, a high and persistent HBsAg-specific humoral immune response was observed at Year 4 (Fig. 5). GMTs increased after each vaccine dose and peaked two weeks post-dose 3 (Week 48), with GMTs >750,000 mIU/mL in all groups. Thereafter, GMTs gradually declined, although there was less than 1 log reduction in

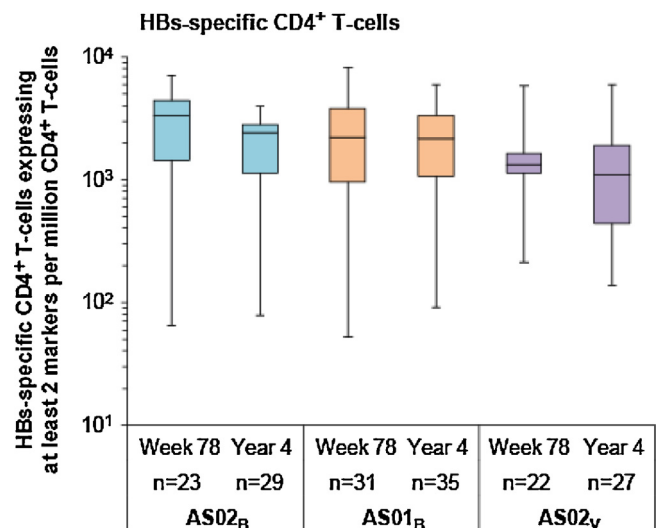


Fig. 2. Frequency of HBsAg-specific CD4⁺ T-cells cells expressing at least two markers (among CD40L, IL2, TNF α and IFN γ) per million CD4⁺ T-cells upon short-term *in vitro* stimulation of peripheral blood mononuclear cells with a pool of peptides covering the entire sequence of the recombinant HBsAg at Week 78 and Year 4. The box represents the upper and lower quartiles; the horizontal line within the box represents the median value; the whiskers represent the minimum and maximum values.

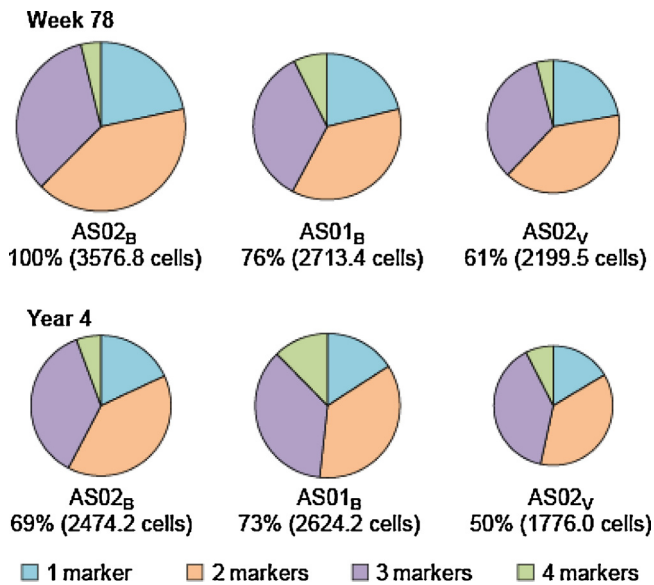


Fig. 3. Mean number of HBsAg-specific CD4⁺ T-cells per million CD4⁺ T-cells in each group expressing 1, 2, 3 or 4 markers (among CD40L, IL2, TNF α and IFN γ) at Week 78 and Year 4 upon short-term *in vitro* stimulation of peripheral blood mononuclear cells with a pool of peptides covering the entire sequence of the recombinant HBsAg. Each pie chart is sized proportionally with regard to the total mean number of HBsAg-specific CD4⁺ T-cells per million CD4⁺ T-cells (mean number shown in parentheses) with the AS02_B group at Week 78 as the reference (100%).

GMTs from post-dose 3 to Year 4 (estimated ratios of decline Week 48:Year 4 GMTs were 2.5–3.2 per year for each group). The rate of decline in antibody titres appeared slower for the period from Week 78 to Year 4 (ratio of 1.4 per year for each group) compared with the period from Week 48 to Week 78 (ratio of 3.2–4.1 per year for each group). At Year 4, all subjects were still seroprotected (antibody titre ≥ 10 mIU/mL) and GMT values were $>100,000$ mIU/mL in all groups, which was higher than the GMT values observed post-dose 2 (Week 6).

3.4. HBsAg-specific memory B-cells

In all groups, the median frequency of peripheral HBsAg-specific B-cells tended to increase from pre-vaccination to two weeks post-dose 2 (Week 6), and further increased two weeks post-dose 3 (Week 48) (Fig. 6). There was good persistence of HBsAg-specific B-cells at Year 4. The median frequencies of HBsAg-specific B-cells per million B-cells at Week 78 and Year 4, respectively, were 2618 and 1804 for AS02_B, 2719 and 1471 for AS01_B, and 3588 and 1491 for AS02_V.

The frequencies of peripheral HBsAg-specific B-cells post-dose 3 (Week 48) in each group did not seem to be correlated with antibody titres at later time points (Week 78 and Year 4), with Pearson's correlation coefficient (r) between 0.3 and 0.5 (Supplementary Fig. 1).

3.5. Impact of DTH challenge on persistence of immune responses

A post hoc exploratory analysis suggested that frequencies of HBsAg-specific CD4⁺ T-cells, CD8⁺ T-cells, memory B-cells, and anti-HBsAg antibody concentrations were within the same range at Year 4, when compared to Week 78 values, for those subjects who had the DTH challenge and those who did not (Supplementary Figs. 2 and 3). The number of subjects who did not have the DTH challenge at Week 78 was small, which limited interpretation of these data.

4. Discussion

GSK's Adjuvant Systems were specifically developed to induce strong and persistent cellular immune responses against vaccine antigens, whilst maintaining a high antibody response and having an acceptable safety profile. The present study was undertaken to evaluate the longer-term persistence of cellular and humoral immune responses against a model antigen (recombinant HBsAg) formulated with three MPL and QS-21-containing Adjuvant Systems, AS02_B, AS01_B and AS02_V. Results up to Week 78 of this study (*i.e.*, 18 months after first vaccination) have been reported previously, and showed that the three formulations induced high levels of antibodies, strong and persistent CD4⁺ T-cell responses for HBsAg, while cytolytic T-cells could be detected in some subjects [8].

We now show that the humoral and cell-mediated immunity elicited by the three MPL/QS-21 vaccine formulations persisted up to 4 years after first vaccination, with no observed differences between the three formulations, although it should be noted that this follow-up was not designed nor powered to make formal comparisons between groups. Previous results from earlier time points showed that the AS01_B formulation induced a stronger and more persistent cell-mediated immune response to HBsAg than the AS02_B or AS02_V formulations [8]. This could be related to different interactions of these adjuvant components with the components of the innate immune response against this antigen in the first weeks after vaccination. Although AS01 also showed improved responses with other antigens against HIV, tuberculosis or malaria, it is still important for each new vaccine to search for the best antigen/adjuvant combination that achieves the desired immune response against this specific pathogen in a specific population [1].

A high and sustained polyfunctional HBsAg-specific CD4⁺ T-cell response (*i.e.*, cells that coproduce more than one cytokine) was induced by all three vaccine formulations following short-term *in vitro* restimulation with HBsAg. Most of the HBsAg-specific CD4⁺ T-cells detected at Year 4 expressed both CD40L and IL2, with a substantial proportion also expressing TNF α and/or IFN γ . This is important as previous studies have shown that a polyfunctional antigen-specific CD4⁺ T-cell response is required for sustained protective immunity against various chronic infections, including tuberculosis, malaria and HIV [20–23].

Similar to earlier time points [8], cytokine-producing HBsAg-specific CD8⁺ T-cells were not consistently detected at Week 78 or Year 4 following short-term *in vitro* restimulation. This suggests that either the frequency of CD8⁺ T-cells induced following vaccination was below the limit of detection, that circulating levels of specific CD8⁺ T-cells may not accurately represent the total specific CD8⁺ T-cell response, or that the vaccine-induced cytolytic activity observed at earlier timepoints [8] was CD4-mediated. The lack of a specific CD8⁺ T-cell response was also noted in other trials with MPL/QS-21 adjuvanted vaccines using purified protein antigens, *e.g.*, malaria (RTS,S), tuberculosis (Mtb72), and HIV-1 (F4) candidate vaccines [14,15,24,25]. This paucity of response was not unexpected since the formulation of soluble antigen with MPL/QS-21-containing Adjuvant Systems was designed to favour HLA-class II mediated antigen presentation to CD4⁺ T-helper cells.

The three MPL/QS-21-adjuvanted vaccines induced a potent and sustained humoral response. The ratios of magnitude of responses at Week 78 *versus* Year 4 suggest more stability for cell-mediated responses than humoral responses. Nonetheless, four years after first vaccination, all vaccine recipients had antibody titres indicative of protection (≥ 10 mIU/mL) and anti-HBsAg antibody levels remained remarkably high (GMT $>100,000$ mIU/mL), predicting an exceptionally long duration of seroprotection. Comparisons across studies should be treated with caution, in part due to differences in vaccination schedules and study populations, but the decline in

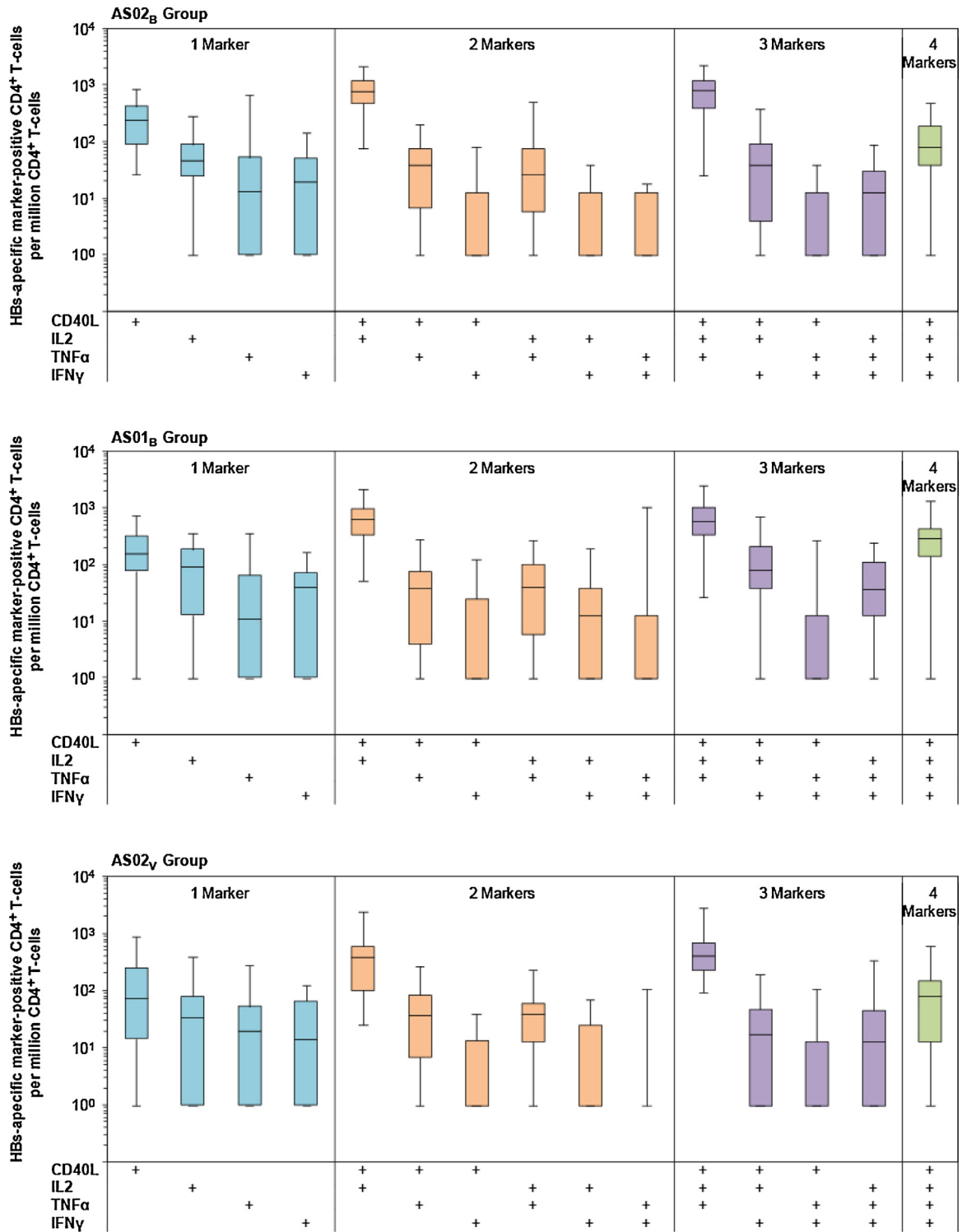


Fig. 4. Frequency of HBsAg-specific CD4⁺ T-cells in each group at Year 4 expressing 1, 2, 3 or 4 markers (among CD40L, IL2, TNFα and IFNγ) upon short-term *in vitro* stimulation of peripheral blood mononuclear cells with a pool of peptides covering the entire sequence of the recombinant HBsAg. Antigen-specific CD4⁺ T-cells were enumerated by flow cytometry following conventional immunofluorescence labelling of cellular phenotype as well as intracellular cytokine production. Results are expressed as frequency of CD4⁺ T-cells expressing markers per million CD4⁺ T-cells. The box represents the upper and lower quartiles; the horizontal line within the box represents the median value; the whiskers represent the minimum and maximum values.

anti-HBsAg antibody titres from post-dose 3 to 4 years after first vaccination with the vaccines formulated with the three MPL/QS-21 Adjuvant Systems appeared less than previously reported with other vaccines which contain the same recombinant HBsAg adjuvanted with aluminium (*i.e.*, *Engerix B*[®] in adolescents [26] and *Twinrix*[®] in young adults [27]).

The kinetics of the HBsAg-specific memory B-cell response followed a generally similar pattern to that of antibody production, with frequencies tending to increase from pre-vaccination to two weeks after the second dose and further increasing two weeks after the third (booster) dose, followed by a gradual decline through Week 78 and Year 4. However, frequencies of memory B-cells after

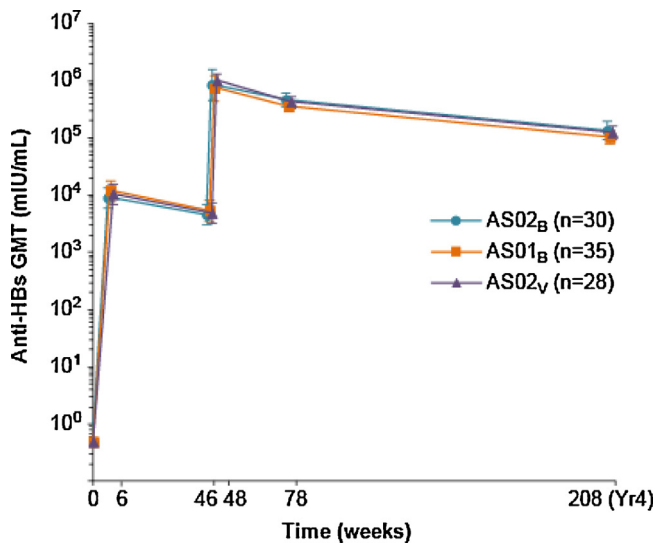


Fig. 5. Antibody response following vaccination at Weeks 0, 4 and 46 with 20 μ g of HBsAg formulated with the AS02_B, AS01_B or AS02_V Adjuvant Systems. Anti-HBsAg antibodies were measured by AxSYM® AUSAB® assay (Abbott Laboratories) and are expressed as geometric mean antibody titre (GMT) with associated 95% confidence interval in mIU/mL. Numbers in parentheses are number of vaccinated subjects at Week 0.

the booster vaccine dose at Week 48 did not seem to be indicative of antibody titres at later time points. At the time of study design, memory B-cells were considered to be the major pool of antibody-secreting plasma cells [28], hence the planned testing and correlation analysis in our study. However, more recent studies have shown that there is only a weak correlation between memory B-cells and serum antibodies (e.g., depletion of memory B-cells in the plasma in patients with autoimmune disease leads to only a minor reduction in antibodies [29]). It is now considered that the major source of stable antigen-specific antibodies in the serum is long-lived plasma cells often surviving in specialised niches in the bone marrow [30,31], rather than memory B-cells recirculating between the peripheral lymphoid tissues [32]. Memory B-cells probably dictate the magnitude and kinetics of the

antibody response following a booster dose (together with CD4⁺ T-cell help) [32].

The late timing of the booster dose at Week 46, together with the immunostimulatory properties of the MPL/QS-21 Adjuvant Systems, may have contributed to the particularly high cellular and humoral responses observed. Another possible important influencing factor is the effect of a DTH skin test performed at Week 78 (involving intradermal injection of 2 μ g purified HBsAg [8]), which may have additionally boosted immune responses. However, no blood samples were taken immediately after this DTH challenge, or in the interval between Week 78 and Year 4, so it was not possible to determine the direct effect of this challenge on immunity. Immune responses have been summarised for the total cohort, regardless of whether the subject received the DTH challenge or not. In an exploratory analysis, immune responses at Year 4 compared to values reported at Week 78 appeared to be in the same range for those subjects who received the challenge and those who did not, although numbers of unchallenged subjects were small, limiting the conclusions that can be drawn.

The main limitation of this study is that the three Adjuvant Systems were evaluated in a specific context using HBsAg as a model antigen in healthy, naïve adults, therefore, findings may not be directly applicable to other scenarios. Although studies with MPL/QS-21 adjuvanted candidate vaccines against complex or chronic diseases show promising results in terms of immune response in HIV and tuberculosis infected subjects [33,34] and demonstrated partial efficacy against malaria [35], there is need for additional studies in terms of protection against those and other diseases in more diverse populations and assess longer term effects. Secondly, we have not tracked any exposure risk factors (transfusions, medical occupations, frequent syringe use) in the trial cohorts. Therefore, we cannot exclude that any natural virus challenges during this follow up period might have influenced the levels of memory B-cell and T-cell activation. Other limitations are that this was an exploratory, descriptive study and no statistical comparisons were done.

In summary, three doses of MPL/QS-21 Adjuvant Systems in combination with HBsAg induced high quality and persistent humoral and cellular immune responses that persisted at least 4 years after first vaccination in healthy adults. These

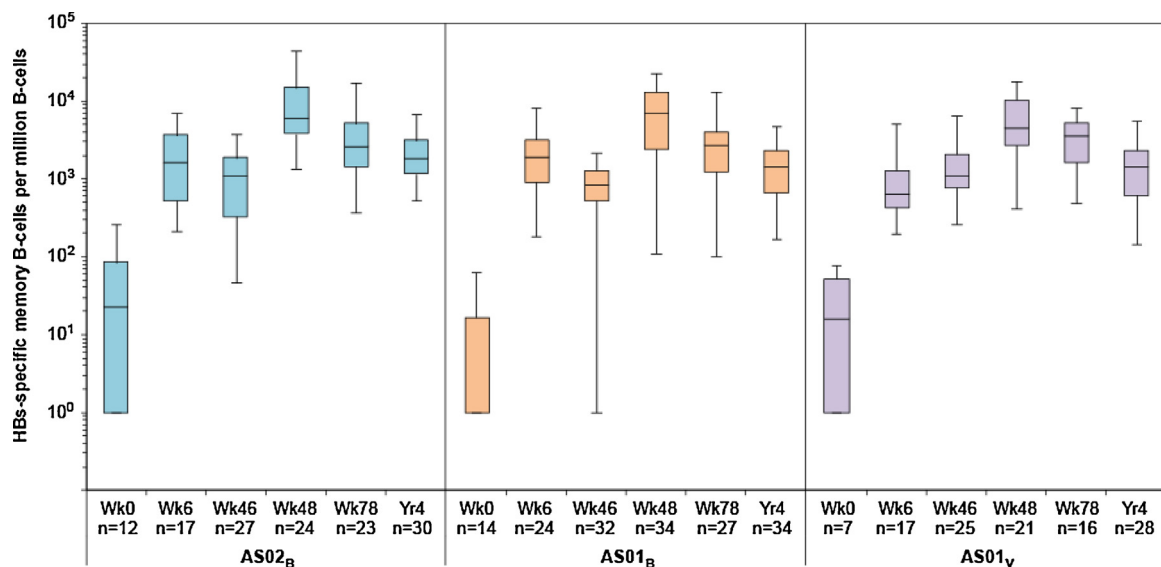


Fig. 6. Frequency of HBsAg-specific memory B-cells following *in vitro* cultivation of peripheral blood mononuclear cells with CpG for 5 days and subsequent detection of antigen-specific plasma cells by ELISPOT. Results are expressed as frequency of HBsAg-specific B-cells per 10⁶ IgG-producing memory B-cells. The box represents the upper and lower quartiles; the horizontal line within the box represents the median value; the whiskers represent the minimum and maximum values. The log-transformation of the frequency of HBsAg-specific B-cells was applied by setting all values of 0 equal to 1. WK: Week; Yr: year.

Adjuvant Systems offer potential for combination with recombinant, synthetic or highly purified subunit vaccines for vaccination against challenging or chronic diseases, as therapeutic vaccines, in immunotherapy, or for subjects with immune dysfunction such as older adults, chronically diseased, or immunocompromised persons. However, additional studies are needed to assess the immunological and clinical effect of these adjuvants in combination with different antigens in different populations against complex diseases such as malaria, tuberculosis or HIV.

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Role of funding source

GlaxoSmithKline Biologicals SA was the funding source and was involved in all stages of the study conduct, analysis and interpretation. GlaxoSmithKline Biologicals SA also took responsibility for all costs associated with the development and publishing of the present manuscript.

Conflict of interest statement

GLR and YH received funding from GlaxoSmithKline group of companies (GSK) via their institute to perform this trial. PVB, MJ, IC, NG, MW, MVM are employees of GSK. PVB, NG, MW, and MVM own restricted shares of GSK. PV was employed by GSK at the time of the study and is now employed by Neovacs S.A. The authors have no other conflict to declare.

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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.2014.10.078>.

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