Vaccine 33 (2015) 4762–4769

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Evaluation of hyaluronic acid-based combination adjuvant containing monophosphoryl lipid A and aluminum salt for hepatitis B vaccine

Se-hee Moon [a], Eui-Cheol Shin [b], Young-Woock Noh [a], Yong Taik Lim [a,*]

[a] Nanomedical Systems Laboratory, SKKU Advanced Institute of Nanotechnology (SAINT), School of Chemical Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Laboratory of Immunology and Infectious Diseases, Graduate School of Medical Science and Engineering, KAIST, 291 Daehak-ro, Daejeon 305-701, Republic of Korea

ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 28 July 2015
Accepted 3 August 2015
Available online 10 August 2015

Keywords:
Adjuvant
Hepatitis B vaccine
Hyaluronic acid
MPLA
Alum

ABSTRACT

Here, monophosphoryl lipid A (MPLA) and aluminum salt (Alum) were introduced into a hyaluronic acid (HA)-based combination vaccine adjuvant for hepatitis B vaccine (HBV). Although Alum is a well-known hepatitis B vaccine adjuvant that induces an enhanced humoral immune response, it cannot induce the cellular immune responses. On the other hand, MPLA has been generally reported to promote IFN-γ production via antigen-specific $CD4^+$ T cells, but it is not water soluble as a result of its long hydrophobic alkyl chains. To this end, water insoluble MPLA could be solubilized in an aqueous solution with the help of HA, which contains many carboxyl and hydroxyl groups that can be used to attach to the hydroxyl head groups of MPLA via hydrogen bonds. Three groups of mice were treated with either hepatitis B surface antigen (HBsAg) alone, HBsAg_Alum complex, or HBsAg_Alum_MPLA/HA complex. The group immunized with the HBsAg_Alum_MPLA/HA complex exhibited a high increase in cellular immune response as well as in humoral immune response relative to the other two groups. The antibody, cytokine and T cell levels were most elevated in the group of mice immunized with HBsAg_Alum_MPLA/HA complex, even at a 1 μg/mice dose, and the magnitude was still maintained even after 8 weeks. Specifically, the antibody value was 120 times larger in mice vaccinated with HBsAg_Alum_MPLA/HA complex than in mice vaccinated with HBsAg_Alum complex designed similar to commercially available hepatitis B vaccine, Engerix B. The cytokine and T cell proliferation levels were 2 times and 6 times larger in mice adjuvanted with HBsAg_Alum_MPLA/HA complex than in those vaccinated with HBsAg_Alum. The results therefore indicate that incorporating MPLA and Alum with HA can be a potent strategy to increase both the magnitude and the persistence of HBsAg-specific immune responses to protect hosts against hepatitis B virus infection.

1. Introduction

Although more than 2 billion people worldwide have been infected with hepatitis B virus (HBV) [1], current prophylactic hepatitis B vaccines based on recombinant hepatitis B surface antigen (HBsAg) have been highly successful in preventing infection and transmission [2–4]. The hepatitis B surface antigen has poor immunogenicity [5], so the HBV vaccine requires the use of adjuvants. These adjuvants act as a delivery system, improving the uptake of antigens by antigen presenting cells (APCs), and allow for persistent release of the antigen, delayed clearance and better exposure to the immune system. Adjuvants are associated with the induction of cellular and humoral adaptive immune responses, and so can also be referred to as immuno-enhancers. They may increase the ability of antigens to activate signaling pathways to control the induction of innate and adaptive immunity, predominantly targeting APCs [6–11]. For over 70 years, aluminum salts (Alum) have been used as adjuvants in human vaccines [12,13]. They act as a depot for vaccine antigen components in order to enhance antigen uptake by APCs. Recent studies have found that antigens absorbed by Alum are presented in a particulate multivalent form and are more efficiently internalized by APCs [14,15]. Alum have been proven to improve the humoral immune response, and to date, this approach toward vaccines is useful in cases where antibodies represent the main protection mechanism against a given disease. However, Alum are often not sufficiently effective when higher levels of antibodies and/or T-cell-mediated immunity, especially T helper-1 (Th1)-biased responses, are required, and for

* Corresponding author. Tel.: +82 31 299 4172; fax: +82 31 299 4119.
E-mail address: yongtaik@skku.edu (Y.T. Lim).

http://dx.doi.org/10.1016/j.vaccine.2015.08.006
0264-410X/

that reason, they are not the optimal choice in several vaccines currently under development where a Th1-type of immune response is needed.

LPS has been found to function as a specific agonist of TLR4, and MPLA is a detoxified derivative of lipopolysaccharide (LPS) isolated from the Gram-negative *Salmonella Minnesota* R595 bacterial strain [16,17] that interacts accordingly via TLR4 [8,18,19]. Several immunogenicity studies in mice, guinea pigs, monkeys and humans have shown MPLA effectively improves the specific antibody and cellular immune response [20]. MPLA activates the TLR4 pathway, which results in an enhanced production of cytokines that leads to the maturation and migration of APCs to the lymph nodes. The innate immune response can be improved by stimulating TLR4 in order to activate NF-κB transcription, which leads to the expression of pro-inflammatory cytokines such as TNF-α and IL-6 [21]. These cytokines enhance the adaptive immune response by inducing APC maturation and simultaneously inhibit the tolerance response by repressing regulatory T cell activity [22]. MPLA has been generally reported to promote IFN-γ production by antigen-specific $CD4^+$ T cells, therefore skewing the immune response toward a Th1 profile [17]. However, MPLA is poorly soluble and is difficult to disperse in an aqueous solution, so various solubility-enhancing techniques have been developed, including formulation in emulsions, formulation in aqueous dispersions containing amounts of surfactants or helper lipids, and inclusion in liposomes [16–18].

Here, we have developed a novel formulation of the HBV vaccine system consisting of the hepatitis B surface antigen and a hyaluronic acid (HA)-based combination adjuvant that contains water-insoluble MPLA and Alum. The MPLA was solubilized in an aqueous solution with the help of HA. HA is a copolymer of N-acetyl-D-glucosamine and D-glucuronic acid, and it is widely distributed throughout connective, epithelial, and neural tissues. HA and its derivatives are viscoelastic, biocompatible compounds that are commonly used in the medical and cosmetic industry, and since HA contains many carboxyl and hydroxyl groups, it can be used to assemble other materials via hydrogen bonds and hydrophobic interactions. In this study, we used HA to successfully disperse MPLA in an aqueous solution through the interaction with the many hydroxyl groups of MPLA. The combined Alum and MPLA/HA vaccine adjuvant is used for a novel formulation of an HBV vaccine system that is expected to induce both humoral and cellular immunity of the hepatitis B surface antigen (Fig. S1).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.08.006

2. Materials and methods

2.1. Antigen, adjuvant, vaccine formulation

To produce the MPLA/HA complex, MPLA (4 mg/ml) was dissolved in dimethylsulfoxide (DMSO), and HA (133 μg/ml) was dissolved in distilled water. 250 μl of the MPLA solution (1 mg, 0.57 μmol) were added to the HA solution (13.3 mg, 0.03 μmol). The mixture was strongly stirred at room temperature for 5 min, then kept in a shaking incubator at 25 °C for 24 h, and finally lyophilized. It was then re-dissolved in 10 ml of distilled water and was dialyzed for 3 days by using a cellulose membrane tube (MWCO 12–14 kDa) in distilled water. The MPLA/HA complex is obtained in powder form by freeze-drying and is then dissolved in PBS by vortexing.

1 μg of HBsAg, kindly provided from LG Life Sciences Ltd. (Daejeon, Korea), is then diluted in PBS to a concentration of 524 μg/ml, and 20 μg of Alum are diluted at a concentration of 20 mg/ml. First, the HBsAg and Alum are inverted before mixing, and then the MPLA/HA complex solution is added. After reacting for 20–30 min, we immunized the mice.

2.2. Mice and immunizations

C57BL/6 mice (Female, 6–8 weeks old) were purchased from KOATECH (Pyeongtaek, Korea) and were maintained under pathogen-free conditions. All of the experiments employing mice were performed in accordance with the Korean NIH guidelines for care and use of laboratory animals. The mice were immunized twice at 2-week intervals using an intramuscular injection in accordance with the experimental group. Before immunization, all of the mice were anesthetized with 200 μl of a 2.5% avertin solution (2,2,2-tribromoehanol-tert amyl alcohol, Sigma). Serum samples were collected from the immunized mice via retro-orbital plexus. The sera were obtained from blood samples by centrifugation at 13,000 rpm for 20 min, and the serum samples were then stored at -20 °C prior to use.

2.3. ELISA (Enzyme-linked immunosorbent assay)

ELISA was conducted to measure the HBsAg-specific antibody response in the immunized mice by determining the total IgG, IgG1, and IgG2c. Briefly, HBsAg (0.5 μg/ml) was absorbed to 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4 °C and was then blocked with PBS containing 2% BSA for 1 h at 37 °C. 100 μl of sera diluted in blocking solution (1:100 for IgG2c and IgG1, 1:300 for IgG) were added and incubated for 1 h at 37 °C, followed by the addition of HRP-conjugated anti-mice IgG, IgG1 or IgG2c at room temperature for 1 h. 100 μl of peroxidase substrate tetramethylbenzidine (TMB) (BD PharMingen) was added for 10 min, and the reaction was stopped with H_2SO_4. The optical density of the samples was measured at 450 nm (OD 450 nm) with a micro-reader (Molecular Devices, Sunnyvale, CA).

2.4. Enzyme linked ImmunoSpot

Interferon-γ (IFN-γ) and interleukin-4 (IL-4) ELISPOT assays were performed in order to detect the HBsAg-specific T cell response in the mouse splenocytes. Mouse IFN-γ and IL-4 kits (BD PharMingen) were used according to manufacturers' instructions. Briefly, the IP-plates were coated with 5 μg/ml of purified rat-anti-mouse IFN-γ or IL-4 antibodies in PBS at 4 °C overnight. The plates were washed three times with PBS, and each plate was then blocked by adding 200 μl of blocking buffer (RPMI with 10% FBS) (Invitrogen) in each well for 2 h at RT. The extracted spleens were rinsed in sterile PBS, and 1.0×10^6 splenocytes per well plated were coated on 96-well plates the day before. The splenocytes were left unstimulated or were stimulated with 5 μg/ml of HBsAg for 60 h at 37 °C. After washing, the samples were incubated with 100 μl of biotinylated rat-anti-mouse IFN-γ or IL-4 antibodies, respectively, and were further incubated at 37 °C for 1 h. After an additional wash, 100 μl of HRP-conjugated streptavidin complex were added to each well in the above dilution buffer at 37 °C for 1 h. The plates were washed, and the spots representing individual IFN-γ or IL-4 producing cells were detected after a 20 min color reaction using an AEC coloring system (Sigma). The spot forming cells/well (SFC) were counted with a CTL ImmunoSpot analyzer (Cellular Technology Ltd., OH, USA).

2.5. T cell proliferation assay

The spleens were rinsed in sterile PBS/0.1% BSA, and isolated spleen cells were pelleted and incubated in an RBC lysis solution. After washing with PBS/0.1% BSA, the cells were incubated for 10 min at room temperature with 1 μM of CFSE. Staining was quenched, and the splenocytes were cultured at 10^6 cells/well in 24-well plates for 5 days at 37 °C in 5% CO_2 with HBsAg, then stained with anti-mouse PE-CD4 and anti-mouse PE-CD8 antibodies, and

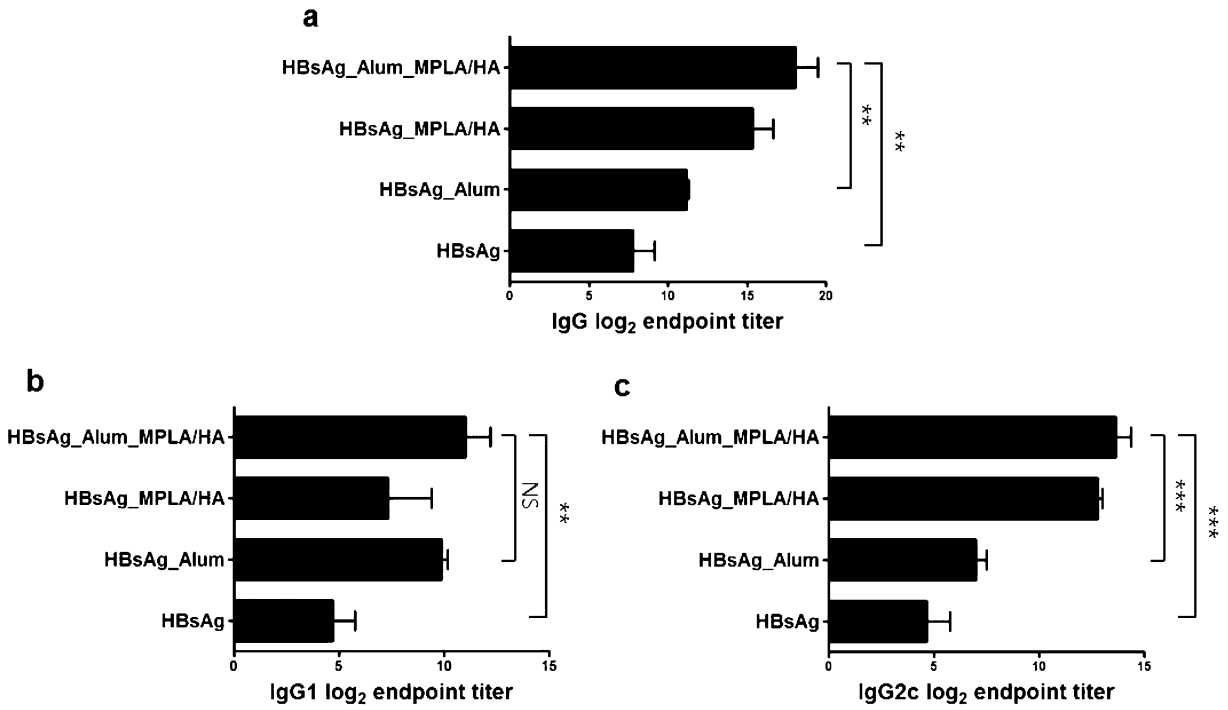

Fig. 1. Alum_MPLA/HA adjuvant enhances anti-HBs antibody titers. Mice ($n = 5$) were immunized intramuscularly twice at a 2-week interval with HBsAg alone or together with Alum, MPLA/HA or Alum_MPLA/HA in 50 μl PBS. Blood samples were collected 2 weeks after the second immunization. (a) Anti-HBs total IgG, (b) IgG1, and (c) IgG2c measured by ELISA (**$p < 0.01$, ***$p < 0.001$, NS: not significant).

analyzed on a FACScanto (BD Biosciences, San Jose, CA, USA). T cell proliferation was expressed as the percentage of divided daughter cells among the total T-cells. Since many daughter cells were generated from each proliferating antigen-specific T cell, this percentage response is proportional but not equal to the actual antigen-specific precursor T cell frequency.

2.6. Statistical analysis

GraphPad Prism 5.0 for Windows was used to draw the graphs and to carry out the statistical analysis (GraphPad Software, San Diego, CA, USA). All results are expressed as the mean differences and were tested for significance using Student's *t*-test, wherein significance is indicated with a p value of <0.05 (*), <0.01 (**), <0.001 (***). $p < 0.05$ is considered to be a significant difference.

3. Results

3.1. MPLA/HA complex development

Alum is well known to have a 'depot effect' and to improve the antibody response, which is part of the humoral immune response, and the addition of MPLA can induce T-cell-mediated cellular immunity [19,23,24]. However, MPLA is hydrophobic, and so it is difficult to incorporate it into a vaccine with antigen and Alum. Here, we used HA to disperse MPLA in an aqueous solution [25]. HA has many carboxyl and hydroxyl groups, and it can be assembled with other materials via hydrogen bonds and hydrophobic interactions. HA in an aqueous solution can stabilize water insoluble MPLA by protecting its hydrophobic groups from the water molecules.

3.2. HBsAg_Alum_MPLA/HA complex enhances anti-HBs antibody responses

The HBsAg antigen was mixed with an adjuvant system composed of Alum and MPLA/HA complex in PBS, and mice were immunized with this HBV vaccine system via intramuscular injections. The antibody response was evaluated via ELISA after immunization with HBsAg (0.5 μg per one mice), Alum (20 μg), and MPLA/HA complex (10 μg/133 μg). When the Alum_MPLA/HA complex was used as vaccine adjuvant, the antibody titers exhibited a 2000-times increase relative to antigen alone and 120 times increase relative to antigen and Alum (Fig. 1a). We also measured the production of IgG1 and IgG2c subtypes, which indicate the Th2 response and Th1 response, respectively. In the case of IgG1, the mice immunized HBsAg_Alum_MPLA/HA complex exhibited a 162-times increase compared to that of antigen only and a 4-times increase compared to antigen plus Alum. In the case of IgG2c, the antibody response showed a 500-times increase when compared with the group immunized with antigen alone and 100-times increase when compared to HBsAg_Alum. The results of the experiment indicate that the HBsAg_Alum_MPLA/HA complex in the hepatitis B virus vaccine induced a high increase in antibody production (Fig. 1b and c).

The capability of the HBsAg_Alum_MPLA/HA complex to enhance humoral immunity at a low dose was tested by serially diluting the antigen dose (0.05–0.5 μg/mice). Fig. 2 shows that the antibody titer value depended on the concentration of the antigen used. The antigen dose was lowered by a factor of 20 relative to the antigen dose in the commercially available Engerix B hepatitis B vaccine. The adjuvant effect was dominant in the mice that had been immunized with HBsAg_Alum_MPLA/HA complex, even at the lowest concentration of antigen, while the effect was not so large in mice immunized with antigen alone or with HBsAg_Alum. It should be noted that the value of induced IgG1 antibody titer was not increased even when Alum was adjuvanted with the diluted amount (0.05 μg) of HBsAg. We investigated effect of the adjuvant on the production of antibody subtypes IgG1 and IgG2c as well as on IgG in general. The antibody response was most dominant in mice immunized with the HBsAg_Alum_MPLA/HA complex. The savings in antigen dosage should be emphasized both for its cost-savings and for the global coverage it allows (Fig. 2).

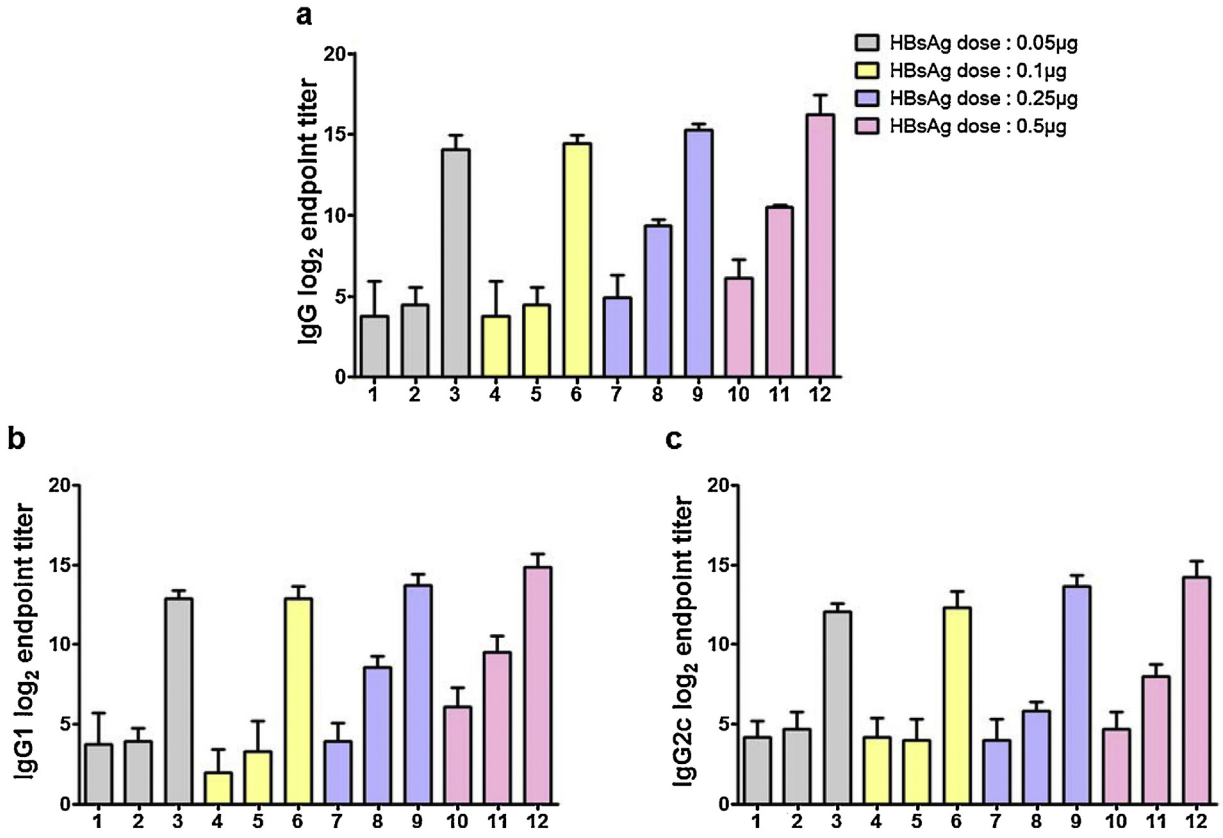

Fig. 2. The effect of the vaccine adjuvant hepatitis B vaccine is dependent on the antigen dose. Mice ($n = 5$) were intramuscularly immunized twice at a 2-week interval with the indicated amount of antigen alone or together with adjuvant in 50 μl PBS. Antibody titer: (a) IgG, (b) IgG1, (c) IgG2c were measured by ELISA [(1, 4, 7, 10); antigen alone, (2, 5, 8, 11); HBsAg_Alum, (3, 6, 9, 12); HBsAg_Alum_MPLA/HA].

3.3. HBsAg_Alum_MPLA/HA complex increases Ag-specific T cell responses

We also examined HBsAg-specific Th1 and Th2 responses after immunization with HBsAg_Alum_MPLA/HA complex. Th1 cells secrete IFN-γ, which activates macrophages and induces the production of opsonizing antibodies by B cells. The Th1 response leads mainly to cell-mediated immunity that protects against intracellular pathogens. Th2 cells secrete cytokines, including IL-4, which induces B cells to produce neutralizing antibodies. Th2 cells generally induce a humoral response that is critical in the defense against extracellular pathogens.

To assess whether the HBsAg_Alum_MPLA/HA complex similarly enhances the HBsAg-specific T cell immunity, splenocytes were isolated from mice to measure the cytokine production using the ELISPOT assay. The potential of the HBsAg_Alum_MPLA/HA complex to induce the anti-HBs IFN-γ-secreting T cells was directly compared to those of antigen only and antigen plus Alum. Mice that had received the HBsAg_Alum_MPLA/HA complex had an approximately 3-times, 5-times increase in the frequency of IFN-γ-secreting T cells relative to those of antigen only and antigen plus Alum. In the case of the IL-4-secreting T cells, we observed a 2-times, 3-times increase in mice immunized with HBsAg_Alum_MPLA/HA complex relative to those of antigen only and antigen plus Alum (Fig. 3). For HBsAg specific T cell proliferation assay, we extracted splenocytes from mice that had been immunized twice, and then splenocytes were labeled with CFSE and cultured with HBsAg for 5 days. HBsAg specific T cell proliferation was determined by FACS to evaluate CFSE-labeled T cell proliferation after they were stained with anti-mouse CD4 and CD8 antibodies [26]. HBsAg_Alum_MPLA/HA complex showed the most potent effect to stimulate both $CD4^+$ and $CD8^+$ T cell proliferation when compared to that of HBsAg alone and HBsAg_Alum complex (Fig. 3).

3.4. HBsAg_Alum_MPLA/HA complex has large effect even at low dose of MPLA

The adjuvants may be added to the vaccine in order to modify the immune to produce a higher amount of antibodies, thus minimizing the amount of material that is injected. We confirm that the HBsAg_Alum_MPLA/HA complex is effective at low dose of MPLA. The doses tested included 10 μg/mice, 5 μg/mice, 2.5 μg/mice or 1 μg/mice of MPLA concentration. As shown in Fig. 4, the mice immunized with HBsAg_Alum_MPLA/HA showed significantly higher levels of IgG than mice immunized using antigen alone or HBsAg_Alum complex groups at a low dose. When compared to the 10 μg/mice dose and 1 μg/mice dose of MPLA, the effect of the antibody response was larger by a factor of 8 in mice immunized with a 10 μg/mice dose of MPLA. In addition, we investigated the effect of the adjuvant on the production of antibody subtypes IgG1 and IgG2c as well as IgG in general. Although the antibody levels are considered to be dependent on the adjuvant dose, the antibody response in mice immunized with HBsAg_Alum_MPLA/HA complex (at a 1 μg/mice dose of MPLA) was similar to that in mice immunized with HBsAg_Alum.

3.5. HBsAg_Alum_MPLA/HA complex helps to maintain immunity during a certain period

A vaccine system that can maintain the immune response for a long period is very important to the vaccine industry.

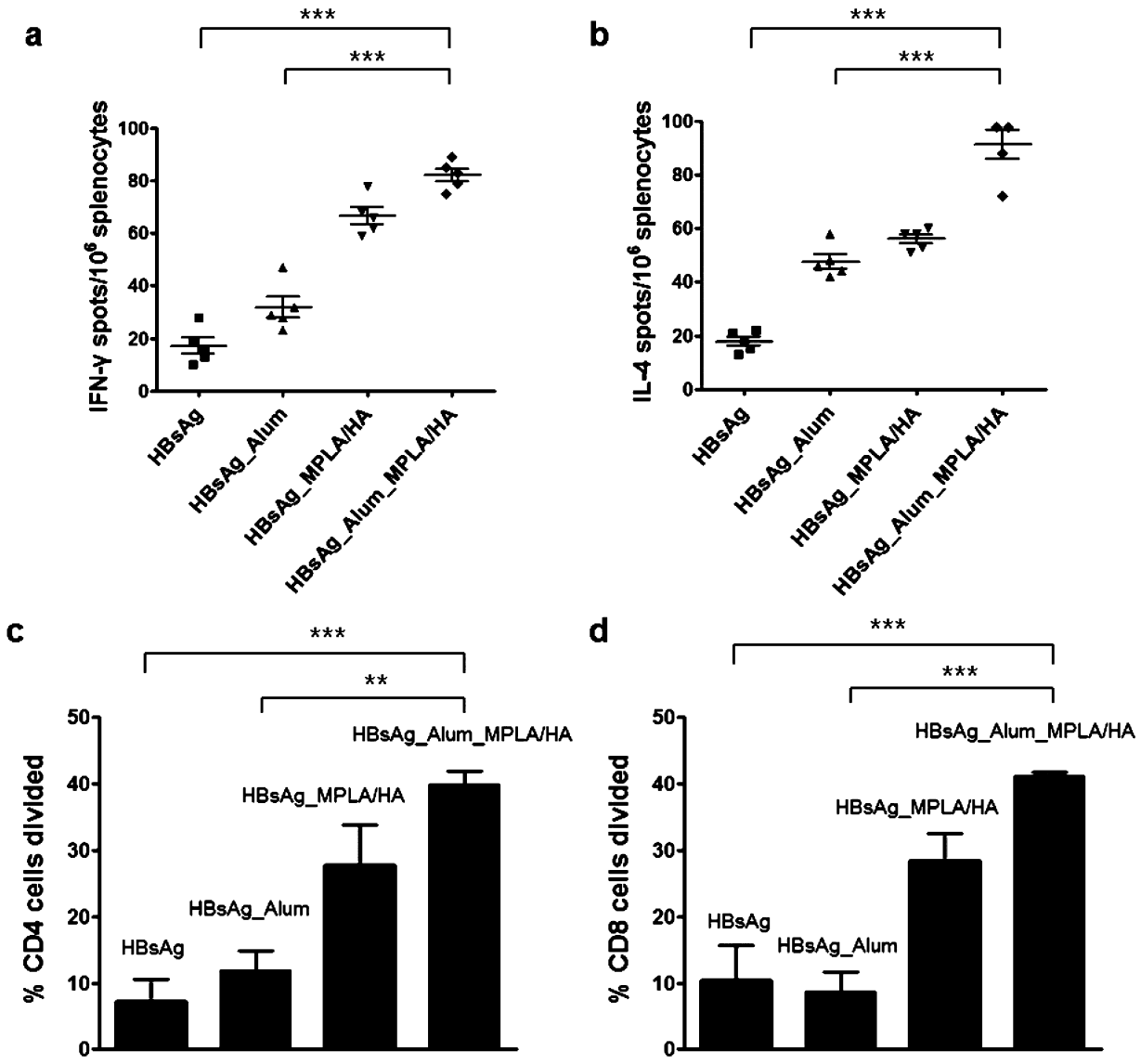

Fig. 3. Alum_MPLA/HA adjuvant enhances T cell responses. Spleen were collected from mice ($n = 5$) immunized with HBsAg alone or together with Alum, MPLA/HA or Alum_MPLA/HA complex in 50 μl PBS. The cytokines (a: IFN-γ, b: IL-4) were quantified via ELISPOT assay. $CD4^+$ (c) and $CD8^+$ (d) T cell proliferation was measured by culturing CFSE-labeled splenocytes with HBsAg for 5 days ($^{**}p < 0.01$, $^{***}p < 0.001$).

Several factors have been shown to contribute to protection for a long period, including immune memory. Memory B and T cells are generated after the initial immune response to vaccination. The contribution of the memory immune response to long-term protection is evidenced by the rapid increase in antibodies after vaccination and by the persistent immunity in individuals after the subsequent decrease in antibody levels.

We have investigated whether a vaccine system composed of HBsAg_Alum_MPLA/HA complex still maintain the adjuvant effect for a certain period. After administering the vaccine in mice twice, the antibody response was analyzed using ELISA (Fig. 5) at 3 and at 8 weeks, and the T cell immunity was analyzed through an ELISPOT assay and CFSE T cell proliferation assay at 8 weeks (Fig. 6a and b). The antibody, cytokine and T cell levels were still high in the mice immunized with HBsAg_Alum_MPLA/HA complex. In the future research, more systematic study on the memory response that can be maintained for longer time and be enhanced with an additional booster, will be conducted.

4. Discussion

The HBsAg based vaccine against HBV has been available since the early 1980s and shown highly protective and safe efficacy. However, 5–15% of normal vaccine recipients considered as non-responders to hepatitis B vaccination as well as patients with a chronic liver disease failed to develop detectable specific antibodies and remained susceptible to HBV even after the hepatitis B vaccination according to the recommended three-dose schedule [27,28]. Thus, there has been a need for a more immunogenic vaccine and vaccine adjuvant for enhanced protective immunity. Aluminum salt is a well-known adjuvant in the hepatitis B vaccine that have been shown to induce an enhanced Th2 response, which control the humoral immune response. However, it induces a weak Th1 response that can be correlated with cytotoxic T lymphocyte (CTL) responses. CTL has been proved to be important in protection from against many infectious diseases as well as cancer [29]. Although not essential for protective immunity against HBV, CTL may nevertheless play an important role for avoiding

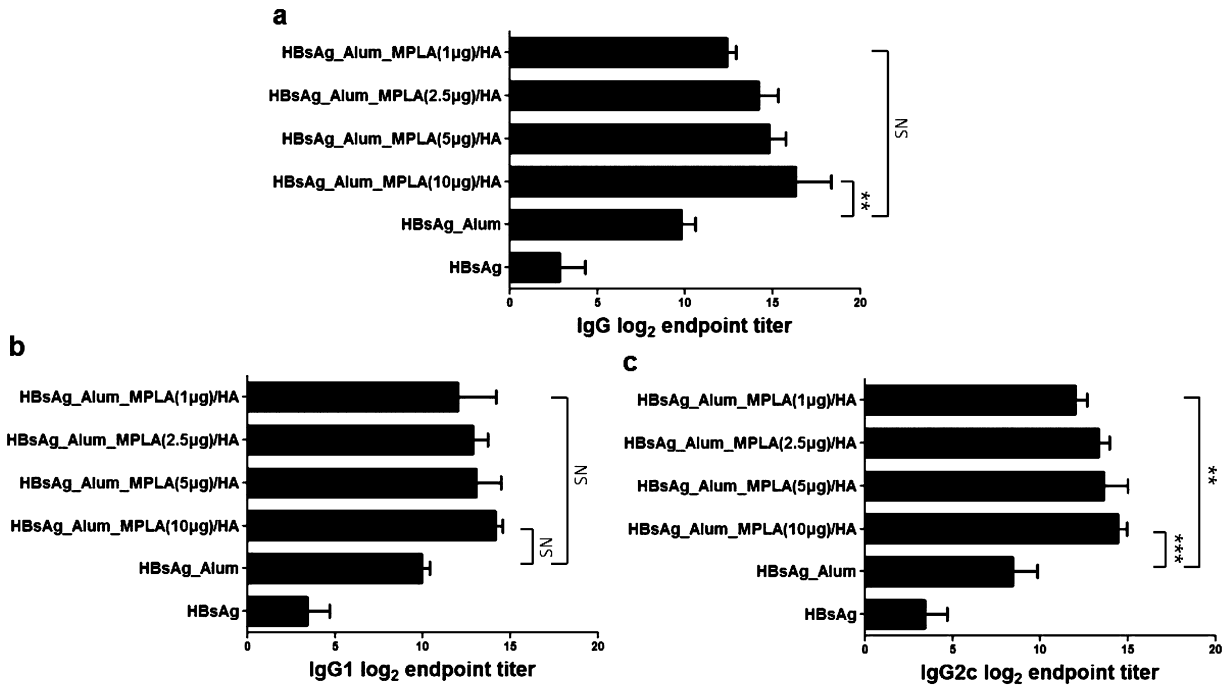

Fig. 4. The effect of the vaccine adjuvant hepatitis B vaccine is dependent on the adjuvant dose. Mice ($n = 5$) were intramuscularly immunized twice at a 2-week interval with the indicated amount of adjuvant in 50 μl PBS. (a) Anti-HBs total IgG, (b) IgG1, and (c) IgG2c measured by ELISA. The adjuvant doses were 10 μg/mice, 5 μg/mice, 2.5 μg/mice and 1 μg/mice according to the concentration of MPLA (**$p < 0.01$, ***$p < 0.001$, NS: not significant).

or overcoming the chronic carrier state [30]. It was reported that a Th2 adjuvant is problematic in the primary protective immune response associated successful production of antibodies in non-responding populations, while an adjuvant inducing a balanced Th1/Th2 response can overcome such non-responsiveness to vaccine in normally non-responder B10.M mice [31]. In particular, the use of alum induced the risk of developing Th2-type diseases such as rheumatoid arthritis, type I diabetes mellitus and systemic lupus erythematosus [32]. This phenomenon, though rare, occurs to an unknown extent in susceptible individuals such as neonates and children. Thus, T cell immune response is needed to facilitate humoral responses and cytotoxic T cell responses for protective immunity.

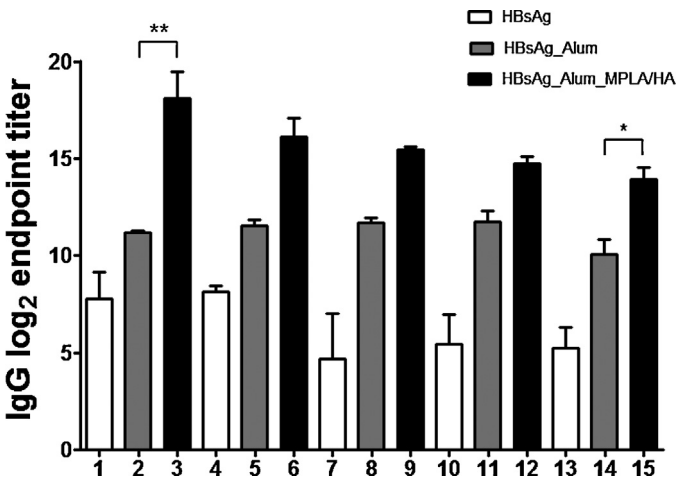

Fig. 5. Alum_MPLA/HA adjuvant sustains anti-HBsAg antibody titers. Mice ($n = 5$) were intramuscularly immunized twice at a 2-week interval with HBsAg alone or together with Alum or Alum_MPLA/HA in 50 μl PBS. Blood samples were collected from 5-weeks to 8-weeks after the second immunization [(1–3); 2-weeks, (4–6); 5-weeks, (7–9); 6-weeks, (10–12); 7-weeks, (13–15); 8-weeks] (*$p < 0.05$, **$p < 0.01$).

In this respect, alum can be combined with monophosphoryl Lipid A (MPLA) to induce the cellular immune response by releasing cytokines [33–36]. However, MPLA is hydrophobic due to its large quantity of hydrophobic alkyl chains. This problem can be addressed through a number of processing techniques developed by the pharmaceutical industry [37,38], such as sonication, which is limited in applicability and is cumbersome. Therefore, an alternative method is needed to diffuse MPLA in water, and to this end, we developed the MPLA/HA complex that can be formed in water by blending at an adequate mass ratio of 10:133. Mice immunized with the HBsAg_Alum_MPLA/HA complex exhibited a high increase cellular immune response and humoral immune response relative to mice vaccinated with HBsAg alone and HBsAg_Alum complex. The effect of the combination was far superior to that of HBsAg_Alum complex, which has similar composition with a commercially available HBV vaccine, Engerix B. In particular, HBsAg_Alum_MPLA/HA complex affected the production of antibodies to achieve a 120-times increase over HBsAg_Alum complex, while cytokine production and T cell proliferation value were 2-times to 6-times higher than those for HBsAg_Alum complex. Our results indicate that MPLA as a TLR4 agonist increased the production of proinflammatory cytokine through signal transmission and stimulated proliferation, differentiation and activation of T cells and B cells. Activated T cells promoted differentiation to cytotoxic T cells and engaged in cellular immunity by releasing cytokines such as IFN-γ [39,40]. Since the HBsAg_Alum_MPLA/HA complex showed a promising contribution to the persistence of an immune response for a long period as well as a reduction in the antigen dose by a factor of 20, it can be used as a novel vaccine formulation against HBV. The results of the experiment suggest that HBsAg_Alum_MPLA/HA complex has promising characteristics for use in hepatitis B vaccines that can supplement commercial hepatitis B vaccines. In this respect, HBsAg_Alum_MPLA/HA complex would be an alternative

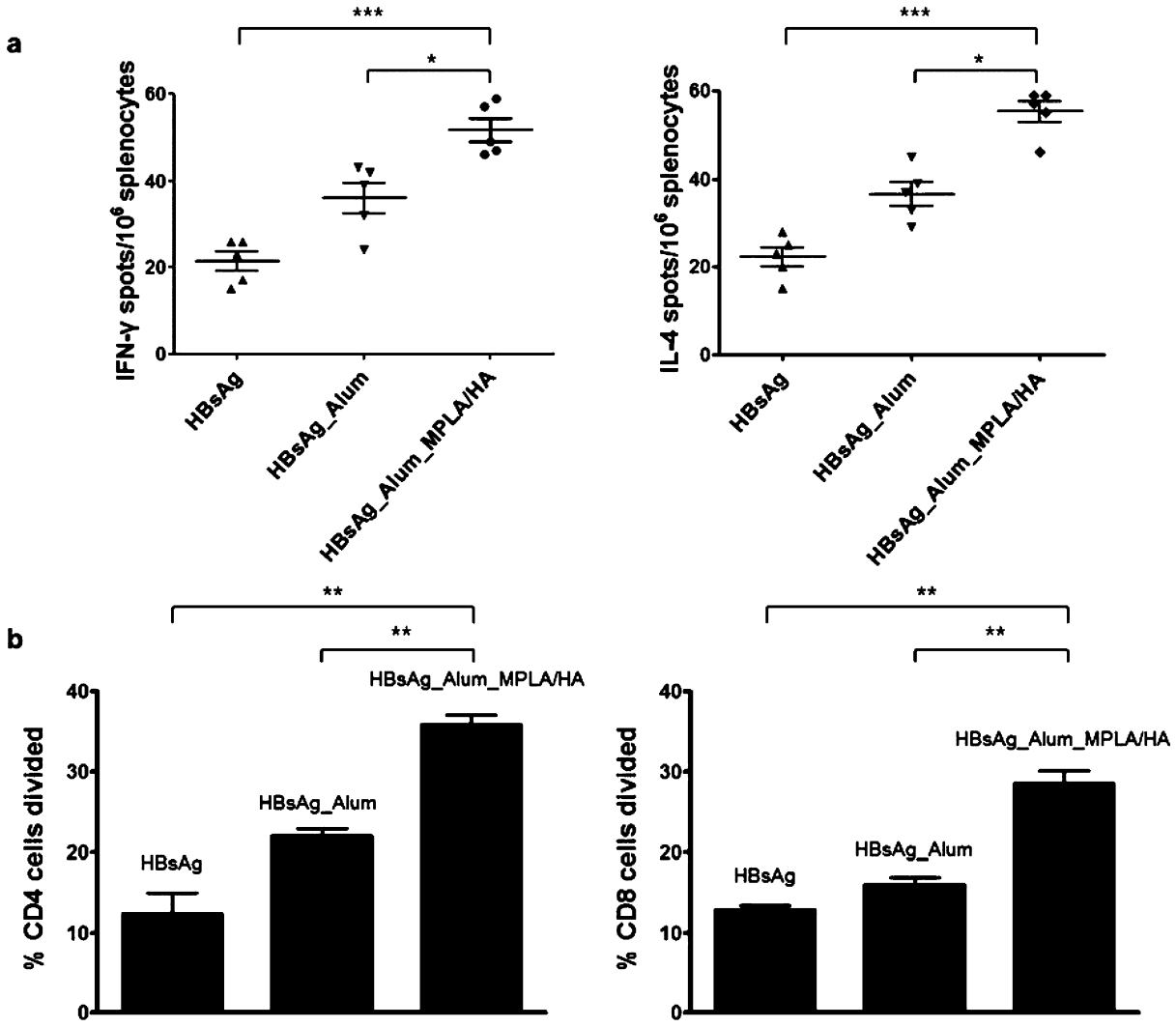

Fig. 6. Alum_MPLA/HA adjuvant sustains the enhancement of Th1, Th2 cytokine secretion. Mice were intramuscularly immunized twice at a 2-week interval. Spleen were collected from mice ($n = 5$) 8 weeks after immunization with HBsAg alone or together with Alum or Alum_MPLA/HA in 50 μl PBS. The cytokines (IFN-γ, IL-4) were quantified via (a) ELISPOT assay and (b) HBsAg-stimulated CD4 and CD8 T cell proliferation measured by culturing CFSE-labeled splenocytes ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS: not significant).

for hepatitis B vaccine non-responders and patients with a chronic liver disease.

Acknowledgement

This work was supported by financial support from the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (grant nos. 2014R1A2A1A10049960, 2012M3A9C6050070).

Conflict of interest statement: The authors declare no conflict of interests.

References

[1] Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 2004;11:97–107.
[2] André FE. Summary of safety and efficacy data on a yeast-derived hepatitis B vaccine. Am J Med 1989;87:S14–20.
[3] Banatvala J, Van Damme P, Oehen S. Lifelong protection against hepatitis B: the role of vaccine immunogenicity in immune memory. Vaccine 2000;19:877–85.
[4] Smith J, Lipsitch M, Almond JW. Vaccine production, distribution, access, and uptake. Lancet 2011;378:428–38.
[5] Saade F, Honda-Okubo Y, Trec S, Petrovsky N. A novel hepatitis B vaccine containing Advax™, a polysaccharide adjuvant derived from delta inulin, induces robust humoral and cellular immunity with minimal reactogenicity in preclinical testing. Vaccine 2013;31:1999–2007.
[6] Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol 2007;5:505–17.
[7] Gupta RK, Griffin Jr P, Chang A-C, Rivera R, Anderson R, Rost B, et al. The role of adjuvants and delivery systems in modulation of immune response to vaccines. Novel strategies in the design and production of vaccines. Springer; 1996. p. 105–13.
[8] Tiberio L, Fletcher L, Eldridge JH, Duncan DD. Host factors impacting the innate response in humans to the candidate adjuvants RC529 and monophosphoryl lipid A. Vaccine 2004;22:1515–23.
[9] Brennan FR, Dougan G. Non-clinical safety evaluation of novel vaccines and adjuvants: new products, new strategies. Vaccine 2005;23:3210–22.
[10] Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol 2004;82:488–96.
[11] Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. Nat Med 2005;11:S63–8.
[12] Garçon N, Segal L, Tavares F, Van Mechelen M. The safety evaluation of adjuvants during vaccine development: the AS04 experience. Vaccine 2011;29:4453–9.
[13] Brewer JM. (How) do aluminium adjuvants work. Immunol Lett 2006;102:10–5.
[14] HogenEsch H. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 2002;20:S34–9.
[15] Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. Vaccine 2005;23:1588–95.

[16] Baldridge JR, McGowan P, Evans JT, Cluff C, Mossman S, Johnson D, et al. Taking a toll on human disease: toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Exp Opin Biol Ther 2004;4:1129–38.
[17] Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 2008;65:3231–40.
[18] Evans JT, Cluff CW, Johnson DA, Lacy MJ, Persing DH, Baldridge JR. Enhancement of antigen-specific immunity via the TLR4 ligands MPL™ adjuvant and Ribi.529. Expert Rev Vaccines 2003;2:219–29.
[19] Martin M, Michalek SM, Katz J. Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. Infect Immun 2003;71:2498–507.
[20] Tomai M, Johnson A. T cell and interferon-gamma involvement in the adjuvant action of a detoxified endotoxin. J Biol Response Mod 1989;8:625–43.
[21] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–95.
[22] Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+ CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033–6.
[23] Keating GM, Noble S. Recombinant hepatitis B vaccine (Engerix-B®). Drugs 2003;63:1021–51.
[24] Garçon N, Morel S, Didierlaurent A, Descamps D, Wettendorff M, Van Mechelen M. Development of an AS04-adjuvanted HPV vaccine with the adjuvant system approach. BioDrugs 2011;25:217–26.
[25] Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review. Vet Med 2008;53:397–411.
[26] Honda-Okubo Y, Saade F, Petrovsky N. Advax™, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses. Vaccine 2012;30:5373–81.
[27] Hadler SC, Francis DP, Maynard JE, Thompson SE, Judson FN, Echenberg DF, et al. Long-term immunogenicity and efficacy of hepatitis B vaccine in homosexual men. N Engl J Med 1986;315:209–14.
[28] Coursaget P, Chotard J, Vincelot P, Diop-Mar I, Yvonnet B, Sarr M, et al. Seven-year study of hepatitis B vaccine efficacy in infants from an endemic area (Senegal). Lancet 1986;328:1143–5.
[29] Rappuoli R. Bridging the knowledge gaps in vaccine design. Nat Biotechnol 2007;25:1361–6.
[30] Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.
[31] Chou H-Y, Lin X-Z, Pan W-Y, Wu P-Y, Chang C-M, Lin T-Y, et al. Hydrogel-delivered GM-CSF overcomes nonresponsiveness to hepatitis B vaccine through the recruitment and activation of dendritic cells. J Immunol 2010;185:5468–75.
[32] Perricone C, Colafrancesco S, Mazor RD, Soriano A, Agmon-Levin N, Shoenfeld Y. Autoimmune/inflammatory syndrome induced by adjuvants (ASIA) 2013: unveiling the pathogenic, clinical and diagnostic aspects. J Autoimmun 2013;47:1–16.
[33] Hernández-Bernal F, Aguilar-Betancourt A, Aljovin V, Arias G, Valenzuela C, Pérez de Alejo K, et al. Comparison of four recombinant hepatitis B vaccines applied on an accelerated schedule in healthy adults. Hum Vaccin 2011;7:1026–36.
[34] Verstraeten T, Descamps D, David M-P, Zahaf T, Hardt K, Izurieta P, et al. Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines. Vaccine 2008;26:6630–8.
[35] Garçon N, Leo O. Innate immunity and vaccine adjuvants: from concepts to the development of a unique adjuvant system AS04 used for the formulation of a human papillomavirus (HPV) vaccine. Curr Cancer Ther Rev 2010;6:126–37.
[36] Giannini SL, Hanon E, Moris P, Van Mechelen M, Morel S, Dessy F, et al. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. Vaccine 2006;24:5937–49.
[37] Garçon N. Preclinical development of AS04. Vaccine adjuvants. Springer; 2010. p. 15–27.
[38] Montomoli E, Piccirella S, Khadang B, Mennitto E, Camerini R, De Rosa A. Current adjuvants and new perspectives in vaccine formulation. Expert Rev Vaccines 2011;10:1053–61.
[39] Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, et al. AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 2009;183:6186–97.
[40] Romagnani S. Th1/Th2 cells. Inflamm Bowel Dis 1999;5:285–94.