**Research Article**

* Astragalus polysaccharides enhance the humoral and cellular immune responses of hepatitis B surface antigen vaccination through inhibiting the expression of transforming growth factor β and the frequency of regulatory T cells

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**Abstract**

*Astragalus* polysaccharides (APS), extracted from the root of *Astragalus membranaceus*, a traditional Chinese medicinal herb, have extensive pharmacological and strong immunomodulatory effects. In this study, the potential adjuvant effect of APS on humoral and cellular immune responses to hepatitis B subunit vaccine was investigated. Coadministration of APS with recombinant hepatitis B surface antigen significantly increased antigen-specific antibody production, T-cell proliferation and CTL (cytotoxic T lymphocyte) activity. Production of interferon-γ (IFN-γ), interleukin-2 (IL-2) and IL-4 in CD4+ T cells and of IFN-γ in CD8+ T cells were dramatically increased. Furthermore, expression of the genes PFP, GrAB, Fas L and Fas were up-regulated; interestingly, expression of transforming growth factor β (TGF-β) and the frequency of CD4+CD25+Foxp3+ regulatory T cells (Treg cells) were down-regulated. Expression of Toll-like receptor 4 (TLR4) was significantly increased by administration of APS. Together, these results suggest that APS is a potent adjuvant for the hepatitis B subunit vaccine and can enhance both humoral and cellular immune responses via activating the TLR4 signaling pathway and inhibit the expression of TGF-β and frequency of Treg cells.

**Introduction**

Hepatitis B is a potentially life-threatening liver disease caused by hepatitis B virus (HBV) infection. It is a global health problem and the most serious type of viral hepatitis (Chisari & Ferrari, 1995). More than 350 million people worldwide are chronic HBV carriers, and 1–2 million people die each year due to the consequences of chronic hepatitis B (Riehmann, 2005). To date, the commercial recombinant hepatitis B surface antigen (HBsAg) vaccine has been widely used, and has become an effective strategy for preventing HBV infection. However, the vaccine primarily induces the antibody response and Th2-biased immune response, but elicits relatively weak cell-mediated immune responses, particularly the antigen-specific CTL response. Therefore, it is unable to clear the virus in the infected cells (Zhang et al., 2009; Geurtsvan et al., 2008).

*Astragalus membranaceus* (Huangqi) is a well-tolerated and nontoxic traditional medicinal herb that is used as a therapeutic agent to treat many diseases in China (Luo et al., 2009; Cui et al., 2003). *Astragalus* polysaccharides (APS), the major component in the root of *A. membranaceus*, has been demonstrated to be a very important immunopotentiator *in vivo* as well as *in vitro* (Zhao et al., 1990; Shimizu et al., 1991). APS have been reported to have profound immunological functions such as suppressing tumor growth, improving humoral and cellular immunity, and regulating the expression of cytokines (Li et al., 2008; Chen et al., 2010). In addition, APS have
been shown to enhance the immune response in immunosuppressed mice (Panhj, 1977). Furthermore, evidence has shown that APS are able to modulate mature of dendritic cells (Shao et al., 2006). However, whether APS as adjuvant influence the host immune response in the context of HBV subunit vaccines remains unclear.

Here we explored the adjuvant effect of APS on HBV subunit vaccine and its mechanism of action in immunized mice. Both humoral and cellular immune responses were enhanced by coadministration of APS. Notably, APS can activate the Toll-like receptor 4 (TLR4) signaling pathway and inhibit negative regulators such transforming growth factor β (TGF-β) and regulatory T cells (Treg cells). This study provides evidence that APS as an adjuvant can efficiently improve the immunogenicity of HBV subunit vaccines via the activation of the innate immune response and inhibition of negative signals.

**Materials and methods**

**Reagents**

*Astragalus* polysaccharide was bought from Nuowei Pharmaceutical Company Limited (Tianjin, China). The recombinant HbsAg (rHbsAg) expressed in CHO cells and the alum adjuvant was kindly provided by North China Pharmaceutical Group Corporation (NCPC, Hebei, China) at 10 μg mL⁻¹. The HbsAg-derived peptides S208–215 (ILSPFLPL; H-2Kb-restricted) were synthesized by GL Biochem Co., Ltd (China). Fluorescent-labeled antimouse monoclonal antibodies, CD8-PE, CD4-PE, IL-4-PE, CD4-FITC, IL-2-FITC and IFN-γ-FITC, were obtained from eBiosciences (San Diego, CA). CFSE was purchased from Fanbo Biochemicals (Beijing, China).

**Animals and immunization**

Adult female BALB/c mice (6–8 weeks old) were purchased from West China Laboratory Animal Center (Chengdu, China) and kept under standard pathogen-free conditions. Mice were randomly divided into five groups (n = 7 each), and immunized intramuscularly on day 0 and 14 with different vaccine formulations (Ragupathi et al., 2008): (1) 1 μg rHbsAg alone, (2) 1 μg rHbsAg plus 500 μg APS, (3) 1 μg rHbsAg plus 10 μg mL⁻¹ alum, (4) 500 μg APS alone and (5) phosphate-buffered saline.

**ELISA**

The serum samples were collected on day 7 after the second immunization and the anti-HBsAg-specific antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (SHICKinghaw Biotech Co. Ltd, Beijing, China). The international unit of total anti-HbsAg antibody was calculated as previously described (Zou et al., 2010).

**T-cell proliferation assays**

Single lymphocyte suspension was prepared from the spleens of mice on day 7 after the second immunization. Cells in RPMI-1640 with 5% fetal bovine serum were incubated in 96-well plates at 37°C with 5% CO₂ and stimulated for 48 h. T-cell proliferation was then carried out by the MTT ((3-(4,5-Di methyl thiazol-2-yl)-2,5-Di phenyl tetrazolium bromide) methods as described previously (Du et al., 2007). OD values were evaluated at 490 nm by a plate reader (Synergy HT, Bio-TEK). Data were expressed as the stimulation index, calculated as the mean reading of triplicate wells stimulated with antigen divided by the mean reading of triplicate wells stimulated with medium.

**Intracellular cytokine staining**

Intracellular cytokine staining was performed as previously described (Wang et al., 2008). Briefly, single T-cell suspension from each group at 1 × 10⁶ cells/100 μL was stimulated in a 96-well plate with HBsAg (5 μg mL⁻¹) for 6 h, and treated with monensin (2 μg mL⁻¹, eBioscience, San Diego, CA) for the last 4 h. Cells were blocked with Fc-Block (BD Phamingen, San Diego) for 30 min. Cells were fixed with 4% paraformaldehyde for 15 min before permeabilization with 0.1% saponin for 10 min. The cells were stained with isotype controls, or double stained with anti-CD8-PE plus anti-IFN-γ-FITC, anti-CD4-PE and anti-IFN-γ-FITC, anti-CD4-PE plus anti-IL-2-FITC, or anti-CD4-FITC plus anti-IL-4-PE for 30 min. The cells were detected by a FACS Calibur and analysed using CELLQUEST PRO Software (BD Bioscience). The frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells was tested with the mouse regulatory T-cell staining kit according the manufacturer’s instructions (eBioscience).

**In vivo cytotoxicity assay**

An in vivo cytotoxicity assay was performed as described previously (Zou et al., 2010). Single suspension cells from naive BALB/c mice were split equally into two portions. One portion as the target cell was labeled with 5 μM CFSE carboxyfluorescein diacetate, succinimidyl ester (Fan-bo biochemicals, Beijing, China; CFSE<sup>high</sup>) after being pulsed with the CTL peptide S208-215 (50 μg mL⁻¹) for 4 h. The other portion as a nontarget control and was labeled only with 0.5 μM CFSE (CFSE<sup>low</sup>). The two portions were mixed in
a 1:1 ratio and injected into immunized mice at 2 × 10^7 total cells per mouse via the tail vein on day 7 after the second immunization. Splenocytes were isolated 4 h later and the CFSE-labeled cells were tested by a FACS Calibur analyzer based on their different CFSE fluorescence intensities. Specific lysis was calculated according to: % specific lysis = [1 – (% specific peptide-loaded target cells/% control peptide-loaded target cells)] × 100%.

**RNA isolation and RT-PCR**

Total RNA was extracted from splenocytes of immunized mice with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was synthesized using Ace reverse transcriptase (Toyobo Co. Ltd, Pudong, Shanghai) with Oligo (dT) 18 primers (the primers for PCR are listed in Table 1). PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining under UV light.

**Statistical analysis**

All experiments were performed at least three times, and the results of one representative experiment are presented. The data were analysed using the two-sided Student’s t-test. Differences were considered to be statistically significant at P < 0.05.

**Results**

**APS coadministration with HBsAg enhances both humoral and cellular immune responses**

To evaluate whether coadministration of APS and hepatitis B vaccine can enhance humoral and cellular immune responses, mice were intramuscularly immunized with rHBsAg alone, rHBsAg + APS or rHBsAg + alum. On day 7 after the second immunization, serum was collected and the total IgG antibody against rHBsAg was analysed by quantitative ELISA. The level of antibody was significantly increased in mice immunized with rHBsAg + APS compared with mice immunized with rHBsAg alone or rHBsAg + alum (Fig. 1a).

For detection of cellular immune response, T lymphocytes were isolated from the immunized mice on day 7 after the second immunization and stimulated with rHBsAg as the specific antigen, concanavalin A as a positive control, bovine serum albumin as a nonspecific control and medium as negative control. The proliferative response was significantly enhanced in the group immunized with rHBsAg + APS compared with other groups (Fig. 1b). T helper (Th) cytokine expression was also detected in CD4^+ T cells by fluorescence-activated cell sorting (FACS). As shown in Fig. 2, mice immunized with rHBsAg + APS induced the highest levels of IL-2, IL-4 and IFN-γ in CD4^+ T cells compared with other groups. As expected, alum increased IL-4 production, but this increase was less than the APS group. These results demonstrated that APS can enhance both humoral and cellular immune responses.

**Effect of APS on cytotoxic response**

The adjuvant effect of APS on antigen-specific cytotoxic response was also detected after the second immunization. An in vivo CTL assay was performed on day 7 after the second immunization. As shown in (Fig. 3a), the

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**Table 1. Target gene primers**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>β-Actin</td>
<td>F:5′-TGGATCCCTGTGGCATCCATGAAAC-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-TAAAACGCAGCTCAGTAAACAGTGCC-3′</td>
</tr>
<tr>
<td>Fas</td>
<td>F:5′-ATGCACACTCTGGATGAAG-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-TTCAAGGGTCATCCTGCTCC-3′</td>
</tr>
<tr>
<td>Fas L</td>
<td>F:5′-AGCTACTGGGGGGCAGTATT-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-ATGCAGGCAATGAGCACCAC-3′</td>
</tr>
<tr>
<td>PFP</td>
<td>F:5′-CCTATGGCAAGCAGCTTTATC-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-ACCTTTGAAATCTGGCACCAC-3′</td>
</tr>
<tr>
<td>GraB</td>
<td>F:5′-GACCCCACTACCTGGCTATT-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-ACCCCATAGACACATCCTC-3′</td>
</tr>
<tr>
<td>TLR4</td>
<td>F:5′-GGTGGCGGTGAGACAAATA-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-AAATCCCTGAAGGCTGGT-3′</td>
</tr>
<tr>
<td>TGF-β</td>
<td>F:5′-ACCTGCAGAAGTCCATGACAT-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-GTTTTCTCATAGTGGGC-3′</td>
</tr>
<tr>
<td>Foxp3</td>
<td>F:5′-ACCCAAGGGGTCAGAACAT-3′</td>
</tr>
<tr>
<td></td>
<td>R:5′-GATCATGGGCTGGTTC-3′</td>
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**Fig. 1.** HBsAg-specific antibody response and T-cell proliferative response. Serum samples from BALB/c mice (n = 7) were collected for ELISA on day 7 after the second immunization. Total anti-HBsAg antibodies were tested by a commercial ELISA kit, and values were determined relative to the standard concentration of anti-HBsAg antibody (a). T cells were isolated from mice of all groups and restimulated with rHBsAg as a specific antigen on day 7 after the second immunization, and proliferation was analysed by the MTT method and expressed as stimulated index (b). *Significant difference between rHBsAg + APS vaccination groups and other immunization groups (P < 0.05).
percentages of antigen-specific lysis of the target cells in
mice immunized with HBsAg, HBsAg + APS or alum
and APS alone were 6.8, 40%, 4.3% and 6.2%, respec-
tively. HBsAg + APS induced the highest CTL activity
among all the groups. The results suggested that APS as
adjuvant could significantly augment antigen-specific CTL
activities in immunized mice.

It is well known that T cytotoxic lymphocytes can
directly clear HBV via effect molecules such as PFP, Gra B,
Fas L and Fas, or by indirectly interfering with the replica-
tion of the virus in infected cells with IFN-γ (Chisari, 1997,
2000). The mRNA levels of these genes were analysed by
semiquantitative reverse transcriptase PCR (RT-PCR) on
day 7 after the second immunization. The production of
IFN-γ in CD8+ T cells was detected by FACS. As depicted
in (Fig. 3c), expression of PFP, Gra B, Fas L and Fas in
the group immunized with rHBsAg + APS was upregulated at
the mRNA level compared with other groups. At the same
time, production of IFN-γ in CD8+ T cells in the group
immunized with rHBsAg + APS was increased compared
with other groups (Fig. 3b and d). Taken together, the data
suggest that APS may be able to eradicate virus by both
lytic and nonlytic cell pathways.

**Effect of APS on TLR-4, TGF-β and Treg cells**

To investigate further how APS as adjuvant modulate the
immune response, mRNA expression of TLR-4 and TGF-β
was analysed by semiquantitative RT-PCR. As shown in
Fig. 4, APS as adjuvant upregulated the expression of
TLR-4, downregulated the expression of TGF-β and
reduced significantly the frequency of CD4+CD25+Foxp3+
Treg cells in mice immunized with rHBsAg + APS, sug-
gest that APS could enhance the immune response by
inhibiting the expression of TGF-β and frequency of Treg
cells and increasing the expression of TLR-4.
Discussion

We have demonstrated that APS is an effective adjuvant for the HBV subunit vaccine, which can improve both HBV-specific humoral and cellular immune responses compared with rHBsAg alone. Most importantly, coadministration of APS and HBV subunit vaccine induced a high level of CTL response and increased IFN-γ production in CD8+ T cells. At the same time, the expression of PFP, Gra B, Fas L and Fas mRNA expression was upregulated. All of these factors play important roles in clearing the virus in HBV carriers. Additionally, higher expression of the innate immune signaling molecule TLR-4, lower expression of TGF-β and lower frequency of Treg cells were observed.

A powerful adjuvant can help antigens to enhance the antigen-specific immune response. Thoelen et al. (2001) demonstrated that the protective antibody was induced in individuals who failed to raise the effective immune response by well-established hepatitis B vaccines when inoculated with SBAS4 as an adjuvant for HBsAg. Our results showed that APS enhanced the level of HBV-specific antibody, T-cell proliferation and the CTL response. An ideal vaccine should be capable of eliciting both strong humoral and cellular immune responses. On
the one hand, the strong antibody response may prevent HBV from entering the host, and neutralize the infected virus in the serum. On the other hand, the cell-mediated immune response plays a critical role in defending and clearing the established HBV infection via cytotoxic activities of CD8+ T cells and natural killer cells. Prince et al. (1997) have reported that chimpanzees immunized with DNA vaccine were protected by the robust cell-mediated immune response in the absence of detectable antibody after intravenous challenge with HBV. In the present study, coadministration of APS and HBV antigen induced both strong cellular and humoral immune responses and may provide protection against HBV.

The Th immune response is important for clearing the virus and preventing its entry into the host. Th1 cells secrete high levels of IFN-γ, which can stimulate the proliferation and division of lymphocytes, facilitate the maturation of dendritic cells and inhibit HBV transcription (Inoue et al., 1989; Bochtler et al., 2008). IL-2 can drive the immunity toward the Th1-biased response to improve the cell-mediated response (Barouch et al., 2000). Th2 cells secrete high levels of IL-4, which can increase antibody production to help the Th2-biased immune response (McKee et al., 2008). In the present study, coadministration of rHBsAg and APS induced high levels of IFN-γ, IL-2 and IL-4 in CD4+ T cells (Fig. 3), indicating that APS as an adjuvant can promote both Th1 and Th2 immune responses.

APS have been widely studied for their immunopotentiating properties, although the underlying mechanism modulating the immune responses remains unclear. Polysaccharides from natural sources such as plants, bacterial and fungi influence the immune system via regulating innate immune signals (Tzianabos, 2000; Brown and Gordon, 2003). Shao et al. (2004) have demonstrated that APS can activate the TLR-4 on macrophages surface in vitro. In the present study, we demonstrated that APS increased the expression of TLR-4 in total splenocytes.

**Fig. 4.** The frequency of Treg cell and mRNA expression of TLR-4 and TGF-β. The frequency of CD25+Foxp3+ in CD4+ T cells was detected on day 3 after the first immunization (a and b). RNA was extracted from splenocytes of immunized mice on day 3 after the first immunization. TLR-4 and TGF-β mRNA expression (c) were detected by semi-quantitative RT-PCR. A gel analysing PCR products is shown as a representative of three independent experiments.
in vivo (Fig. 4), suggesting APS activate the innate immune system through the TLR-4 signaling pathway. We aim now to detect which type of cells increased the expression of TLR-4. It is well known that removal of any negative signals is helpful in regulating the immune system. Yoo et al. (1996) demonstrated that TGF-β, as an immunosuppression factor, was most often observed at higher levels in liver cells from patients with chronic hepatitis, cirrhosis and liver cancer. Foxp3, the forkhead/winged helix transcription factor, is crucial for the development and function of CD4+CD25+ Treg cells, and plays a regulatory role in immunologic suppression (Kao et al., 2008; Di Nunzio et al., 2009; Kubota et al., 2010). Remarkably, APS as an adjuvant can inhibit the expression of TGF-β and the frequency of CD4+CD25+ Foxp3+ (Fig. 4). These results indicated that APS enhanced the immune response via inhibiting negative signals.

In summary, our data showed that APS can be used as an effective adjuvant for enhancing both humoral and cellular responses to the hepatitis B vaccine via activating the innate signaling pathway and inhibiting negative signals. This strategy may provide a powerful prophylactic or therapeutic candidate vaccine for HBV infection.

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Authors’ contribution

X.D., X.C. and B.Z. contributed equally to this work.

References


