Mushroom lectin overcomes hepatitis B virus tolerance via TLR6 signaling

Meina He, Dan Su, Qinghong Liu, Wenjuan Gao & Youmin Kang

Currently, chronic hepatitis B virus (HBV) infection remains a serious public health problem in the world. Recombinant HBV vaccine, as a preventive strategy against HBV infection, generates high antibody level, but it is not effective to activate innate and cellular immunity for chronic HBV infection therapy. Lectins from mushroom are natural and active proteins which have been shown important biological functions. However, little is known about the immunological mechanism engaged by mushroom lectins. Here we report that, lectin from *Pleurotus ostreatus* (POL) stimulated innate response by activating Toll-like receptor 6 signal pathway of dendritic cells. Subsequently POL enhanced HBV specific antibody level and follicular helper T cells response which overcame HBV tolerance in transgenic mice. This study suggests a novel mechanism for POL acting on immune response and a therapeutic approach to break HBV tolerance.

Chronic hepatitis B virus (HBV) infection is a serious disease that causes public health problems worldwide. Accumulated data have shown that a recombinant HBV vaccine with an alum adjuvant generates high antibody level and is a promising strategy for activating immune response and protecting against HBV infection in humans. Alum (Al) is the most widely used adjuvant in humans because it primarily elicits a T helper 2 (Th2) cell mediated response and has a good safety record. For almost one century, alum has been the only adjuvant approved and licensed for human vaccine by the U.S. Food and Drug Administration. However, HBV vaccine containing alum as an adjuvant and recombinant HBV surface antigen (HBVsAg) are not effective for chronic HBV infection since it does not elicit an effective cellular immune response and has no therapeutic effect in chronic HBV carriers. Currently available therapies fail to control viral replication in most patients.

Several methods, including adjuvants, have been suggested to enhance the immune response generated by recombinant HBV vaccine. Levamisole is an antihelminthic drug that stimulates T-cell response. In one study, dialysis patients showed a significant improvement in immune response to HBV vaccine when levamisole was used as an adjuvant; however, the limited number of patients in the study limits the conclusions that could be drawn. A combination of levamisole and an alum adjuvant has been shown to synergistically enhance the immunogenicity of HBVsAg. In another study, HBV vaccine with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant elicited increased patient response rates compared with HBV vaccine alone. Administration of GM-CSF prior to vaccination with recombinant HBV vaccine produced high IgG level and stimulated CD8+ T cellular response in HBV-transgenic mice. A formulation comprising recombinant HBV and a CpG oligonucleotide (1018 ISS) has been shown to induce a robust humoral and cell mediated immunity against HBV. Heat shock protein gp96 enhanced immune responses and potentiates the anti-HBV activity in BALB/c and transgenic mice.

Lectins induce cell agglutination and have been shown to be possessed in important biological processes. Lectins are abundant in mushrooms, and a variety of lectins have been isolated from edible mushrooms. Although several mushroom lectins have been purified and characterized, only some have been shown to possess immunological activity. Some mushroom lectins showed mitogenic activities towards mouse T cells. Lectin from *Pleurotus ostreatus* (POL) has high antitumor activity. Our previous study showed that POL as an adjuvant in an HBV DNA vaccine activated strong Th2 and cytotoxic T cell responses.

Innate immunity plays a major role in host defense during the early stages of infection. The first step in innate immunity is the recognition of microbes by receptors including toll-like receptors (TLRs). C-type lectins are a type of pattern recognition receptor, which mostly recognize carbohydrate structures in pathogens. TLRs are...
family of ten microbe-recognition receptors that are important to mediate effective innate immune response. TLRs generate intracellular signals with the potential to elicit inflammatory responses. Little is known about the effect of mushroom lectins on innate immunity. In this study, we report for the first time the activation of innate immunity by POL for treatment of chronic HBV infection.

Results

POL increased HBV-specific cellular immune response in immunized C57BL/6 mice. C57BL/6 were randomly divided into five groups (n = 9 per group). Mice were injected intramuscularly with 2 µg recombinant HBVsAg vaccine antigen (VAg group), 2 µg recombinant HBV vaccine (Vac group), 2 µg recombinant HBVsAg vaccine antigen and 1 µg POL (POL/VAg group), 2 µg recombinant HBVsAg vaccine and 1 µg POL (POL/Vac group). A control group was injected with saline. The mice were immunized on day 0 and boosted on days 14 and 28. All experiments were repeated three times. The injection sites exhibited no erythema or edema, and all mice appeared healthy after the injections. To check the cellular response stimulated by POL, splenocytes of immunized mice were prepared for T-cell proliferation analysis by MTT (demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Fig. 1A) and CFSE (demonstrated by 5,6-carboxyfluorescein diacetate, succinimidyl ester) staining (Fig. 1B). The a-CD3 monoclonal antibody was used as a positive control and showed high stimulation index (SI) while bovine serum albumin (BSA) was used as a negative control and showed low stimulation index (SI). The SI of the POL/VAg group was significantly higher than that of the VAg (p < 0.05) and Vac (p < 0.05) groups. Furthermore, the SI of the POL/Vac group was higher than that of the VAg (p < 0.01), Vac (p < 0.01) and POL/VAg (p < 0.05) groups (Fig. 1A). Similarly, the percentage of proliferative cells (R1) in the POL/Vac group was higher than those in the other groups (Fig. 1B).

Cytokines play important roles in the stimulation of immune response. To test the cytokine profiles of CD4+ T-cells, splenocytes of immunized C57BL/6 mice were prepared and intracellularly stained for flow cytometry analysis. The CD4+ T cells were gated and CD4+IFN-γ+ CD4+ IL-4+ or CD4+IL-21+ cells were counted relatively to total CD4+ T cells (Fig. 2A). The POL/VAg group exhibited significantly elevated levels of Th1 cytokines (CD4+IFN-γ+) compared with the VAg group (p < 0.05), but showed no significant difference compared with
the Vac or POL/Vac groups (Fig. 2B). Analysis of Th2 cytokine levels (CD4+ IL-4+) revealed that the Vac group produced significantly higher levels than the VAg (p < 0.01) and POL/VAg (p < 0.01) groups; the POL/VAg group produced significantly higher levels than the VAg group (p < 0.01); and the POL/Vac group produced significantly higher levels than the VAg (p < 0.01) and POL/VAg (p < 0.05) groups; but there was no significant difference between the POL/Vac and Vac groups (Fig. 2B). Analysis of IL-21 in CD4 T-cells revealed that the POL/VAg group produced significantly higher levels than the VAg (p < 0.05) and Vac (p < 0.05) groups; and the POL/Vac group produced significantly higher levels than the VAg (p < 0.01) and Vac (p < 0.01) groups, but there was no significant difference between the POL/Vac and Vac groups (Fig. 2B).

**POL elicited a follicular helper T cell (Tfh) response in immunized C57BL/6 mice.** As a Tfh cell-secreted cytokine, IL-21 is one of the most important B-cell stimulators and has functional roles in humoral immunity. Because POL increased the level of IL-21 expression in CD4 T-cells (Fig. 2), IgG levels, Tfh cells and germinal center (GC) B-cells of immunized C57BL/6 mice were analyzed. Analysis of HBVsAb levels revealed that the Vac group exhibited significantly higher levels than the VAg (p < 0.01) and Vac (p < 0.01) groups; and the HBVsAb level in the POL/Vac group was the highest among all the groups (Fig. 3A). For Tfh cell evaluation, CD4+ T-cells were gated and Tfh cells (CD4+ CXCR5+ PD-1+) were analyzed. The percentage of Tfh cells was significantly higher in the Vac group than in the VAg group (p < 0.01); significantly higher in the POL/Vag group than in the VAg group (p < 0.01); and significantly higher in the POL/Vac group than in the VAg and Vac groups (p < 0.01); but there were no significant differences in Tfh cell percentage among the Vac, POL/Vag and POL/Vac groups (Fig. 3B,C). For GC B-cell evaluation, B220+ cells were gated and GC B-cells (B220+ CD95+ GL-7+) were analyzed. The percentage of GC B-cells was significantly higher in the Vac group than in the VAg group (p < 0.01); significantly higher in the POL/Vag group than in the VAg and Vac groups (p < 0.01); and significantly higher in the POL/Vac group than in the VAg and Vac groups (p < 0.01); but there was no significant difference in GC B-cell percentage between the POL/Vag and POL/Vac groups (Fig. 3D,E).
POL stimulated the TLR6 signaling pathway in dendritic cells (DCs) of immunized C57BL/6 mice. The major histocompatibility complex (MHC) molecules and co-stimulators play essential roles in DC maturation and innate immunity. To investigate DC maturation, splenocytes of immunized mice were stained with anti-mouse CD11c, CD40 and CD80 mAbs. CD40 and CD80 expression in DCs were increased in the POL/Vac group compared with other groups (Fig. 4), but there were no differences in MHCII or CD86 expression in DCs among all the groups (data not shown).

The first step in innate immunity is the recognition of microbes by different receptors. C-type lectins are pattern recognition receptors that mostly recognize carbohydrate structures in pathogens. C-type lectins, including mannose receptor (CD206), DCs-specific intercellular adhesion molecule-3-grabbing non-integrin, CD209 (DC-SIGN) and DCs associated C-type lectin 1 (Dectin-1, a specific receptor for β-glucans), activate the host innate immune system. To assess whether POL can activate C-type lectins, splenocytes of immunized mice were stained with anti-mouse CD11c, CD206, CD209 and dectin-1 antibodies. There were no differences in CD206, CD209 or dectin-1 expression in DCs among all the groups (data not shown).

TLRs are a family of ten microbe-recognition receptors that are important to effective innate response. TLRs generate intracellular signals that have the potential to elicit inflammatory responses. To assess whether TLRs can be activated by POL, quantitative PCR to evaluate TLR levels (TLRs 1–9, 11 and 12) were performed. TLR6 mRNA expression in the POL/VAg and POL/Vac groups was significantly upregulated, compared with in the VAg (p < 0.01) and Vac (p < 0.01) groups, but there was no significant difference between POL/VAg and POL/Vac groups (Fig. 5A). There were no differences of other TLRs expression among all the groups (data not shown).
To confirm upregulation of TLR6, TLR6 protein levels were measured by flow cytometry. TLR6 expression was increased in the POL/VAg and POL/Vac groups (Fig. 5B). To examine the TLR6 signaling pathway, western blotting was performed to evaluate expression of the adaptor protein MyD88 (demonstrated by myeloid differentiation primary response gene 88) and the signal molecules IRAK1 (demonstrated by interleukin 1 receptor associated kinase 1), TRAF6 (demonstrated by TNF Receptor-Associated Factor 6), p-IκB (demonstrated by...
phosphorylated inhibitor of NF-κB) and p-NF-κB (demonstrated by phosphorylated Nuclear factor-kappa B). Expressions of these molecules were upregulated in the POL/V Ag and POL/Vac groups compared with other groups (Fig. 5C). Similarly, IL-1β mRNA expression was significantly upregulated in the POL/Vac group compared with in the other groups (p < 0.01). IL-1β mRNA expression was significantly increased in the POL/V Ag and Vac groups compared with the V Ag group, but there was no significant difference between the POL/V Ag and Vac groups (Fig. 5D).

Therapeutic effect of POL in HBV-transgenic mice. The HBVAg-transgenic mouse constitutively produces large amounts of HBsAg in the liver, so it is considered a preclinical model for evaluating specific immunotherapy for HBV infection7. To confirm the immune effect of POL, HBVAg-transgenic mice were treated with POL/V Ag and POL/Vac. Untreated, and V Ag- and Vac-treated HBVAg-transgenic mice were used as controls. Seven days after the third treatment, sera were collected and HBsAg and HBVAb levels were evaluated. Among the groups, the serum HBVAb level was highest in the POL/Vac group (p < 0.01) (Fig. 6A). Serum HBVAb levels were higher in the POL/V Ag and Vac groups than in the V Ag group (p < 0.01), but there were no differences between the POL/V Ag and Vac groups. Among all of the groups, the serum HBVAb level was lowest in the POL/Vac group (p < 0.01). The serum HBVAg level in the POL/V Ag group was lower than those in the V Ag and Vac groups. There was no difference in serum HBVAg level between the Vac and Vag groups (Fig. 6B). Similarly, the POL/Vac group exhibited the least lymphocyte infiltration (Fig. 6C) and the lowest HBVAg expression in liver (Fig. 6D). The HBVAg expression level in liver was lower in the POL/V Ag group than in the V Ag and Vac groups, consistent with serum HBVAg levels (Fig. 6D). As shown in Fig. 7, the infiltrated lymphocytes of livers were analyzed by flow cytometry. The results showed that the infiltrated CD8+ T and B220+ B cells were decreased in POL/Vac group compared with other groups. There was no difference in CD4+ T cells infiltration between the Vac and Vag groups (Fig. 7).

POL overcame tolerance by activating innate response in HBV-transgenic mice. In HBV-transgenic mice, HBsAg as a self-antigen cannot stimulate specific immune response. To confirm the effect of POL on cellular immune response in HBV-transgenic mice, splenocytes of treated mice were intracellularly stained with anti-mouse CD4, IL-4 and IL-21 antibodies for cytokine analysis. Compared with in the V Ag group, IL-4 expression was increased in the Vac (p < 0.05), POL/V Ag (p < 0.05) and POL/Vac (p < 0.01) groups, but
there were no significant differences among these three groups. The POL/Vac group exhibited the highest level of CD4 T-cell IL-21 expressions. The POL/V Ag group produced more IL-21 than the V Ag (p < 0.05) and Vac (p < 0.01) groups (Fig. 8A). The results for IL-4 and IL-21 in CD4 T-cells were consistent with those for serum.

Because POL affected Tfh and GC B-cells in C57BL/6 mice, the same experiments were performed in HBV-transgenic mice. In these mice, compared with the V Ag group, the Vac (p < 0.01), POL/V Ag (p < 0.01) or POL/Vac (p < 0.01) group exhibited higher levels of Tfh cell stimulation, but there were no differences among these three groups (Fig. 8B). The same results were observed for GC B-cells.

To confirm the effect of POL on innate immunity in HBV-transgenic mice, the TLR6 signaling pathway was analyzed. TLR6 mRNA expression levels were significantly higher in the POL/V Ag and POL/Vac groups than in the V Ag (p < 0.01) and Vac (p < 0.01) groups (Fig. 8C), consistent with the findings in C57BL/6 mice. IL-1β mRNA expression was upregulated in POL/Vac group compared with other groups (p < 0.01). There were no differences among the V Ag, Vac and POL/V Ag groups (Fig. 8D).

Discussion

HBV is an infectious disease that causes chronic hepatitis and liver cirrhosis globally. A recombinant HBV vaccine with an alum adjuvant is widely used for protection against HBV infection3. However, this vaccine primarily induces a humoral response that is not effective for eradication of chronic HBV infection 32. Many approaches have been developed to enhance the immune response to protein vaccines, particularly with respect to the choice of adjuvant1. We describe herein the use of a novel adjuvant combined with a recombinant HBV vaccine against chronic HBV infection. Our results show that POL, a lectin from Pleurotus ostreatus, activated the TLR6 signaling pathway and elicited HBV-specific Tfh responses for chronic HBV infection treatment.

Studies have shown that lectins play important roles in many biological activities 17. Many mushroom lectins have been isolated and studied for their functions23. POL showed high antitumor activity against sarcoma and hepatoma in mouse models26, 33. Here, we found that POL combined with a recombinant HBV vaccine stimulated HBV-specific T-cell proliferation (Fig. 1). Tfh cells are important in humoral immunity, and IL-21 is important for Tfh cell generation 30, 34. In this study, we found that POL stimulated IL-21 production, and activated Tfh cells and GC B-cells, which contributed to an increase in HBVsAg-specific IgG levels (Fig. 3). These results demonstrate the effect of POL on HBV-specific humoral immune responses.

Innate immunity is important in host defense during the early stages of HBV infection. As antigen presenting cells, DCs are essential for the initiation of innate immunity and activation of adaptive immunity 36. In this study, we found that POL stimulated DCs maturation by increasing CD40 and CD80 expression (Fig. 4). DCs recognize different microbes via different receptors29. TLRs are important receptors and generate intracellular signals...
for effective innate response. Little is known about the effect of mushroom lectin on innate immunity. Here, we report for the first time that POL activated the TLR6 signaling pathway by upregulating expression of TLR6 and its related signal molecules, MyD88, IRAK1, TRAF6, IκB, NF-κB, and also of the inflammatory factor IL-1β (Fig. 5).

Figure 8. POL activated Th and innate responses in treated HBV-transgenic mice. (A) 7 days after the 3rd treatment, the splenocytes of treated mice were prepared. The samples were stimulated with Cell Stimulation Cocktail (eBioscience, San Diego, CA) and treated with Protein Transport Inhibitor Cocktail (eBioscience, San Diego, CA). The samples were intracellularly stained with anti-mouse CD4-FITC/IL-4-PE and anti-mouse CD4-FITC/IL-21-PE for cytokine productions in CD4 T cells by flow cytometry. The CD4+IL-4+ cells or CD4+IL-21+ cells were counted relatively to total CD4+ T cells. (B) 7 days after the 3rd treatment, the splenocytes of treated mice were prepared. The samples were stained with anti-mouse CD4-APC-Cy7/CXCR5-V450/PD-1-PE mAbs for Th cells analysis and stained with B220-PE-Cy5/CD95-APC/GL-7-FITC for GC B cells analysis by flow cytometry. The Th cells and GC B cells were counted relatively to total CD4+ cells or total B220+ cells. (C) 3 days after the 3rd treatment, total RNA from spleens was extracted and qPCR were performed for TLR6 expression. (D) 3 days after the 3rd vaccination, total RNA from spleens was extracted and qPCR were performed for IL-1β expression. Shown in each panel is 1 of at least 3 experiments with similar results. Bar, mean and SD from 3 independent experiments, each using at least three mice per group (n = 3); *p < 0.05; **p < 0.01.
The HBVsAg-transgenic mouse is considered a preclinical model of HBV infection because it constitutively expresses HBVsAg in the liver and mimics healthy human chronic HBVsAg carriers. Many approaches to treat chronic HBV infection have been studied. In one study, levamisole used as an adjuvant in a recombinant HBV vaccine improved specific immune response in dialysis patients; however, the small number of study participants limited the conclusions that could be drawn. In another study, GM-CSF increased response rates among HBV-infected patients when it was combined with a recombinant HBV vaccine. 1018 ISS elicited seroprotective antibodies with fewer vaccinations than recombinant HBV vaccine only in a Phase III clinical trial. In another study, inclusion of alum in a recombinant HBV vaccine stimulated Th2 responses, but had no therapeutic effect on chronic HBV infection. Clearing persistent extracellular antigen of HBV could be an immunomodulatory strategy to reverse tolerance for an HBV infection therapy. In this study, HBVsAg-transgenic mice were treated with POL/Vac; this combination showed a promising therapeutic effect. HBV-specific IgG levels were increased and HBVsAg levels were decreased significantly in treated HBVsAg-transgenic mice. Similarly, lymphocyte infiltration, particularly of CD8 T- and B-cells in the liver, was significantly decreased in treated HBVsAg-transgenic mice (Fig. 6). In POL-treated HBVsAg-transgenic mice, POL stimulated Th responses (Fig. 8B) consistent with those observed in immunized C57BL/6 mice (Fig. 6). The TLR6 signaling pathway was also activated in POL-treated HBVsAg-transgenic mice (Fig. 8C), consistent with findings in immunized C57BL/6 mice (Fig. 6). In summary, the results demonstrated that POL may interact with TLR6 and elicit HBV specific immune response. TLR6 expression and downstream signal molecules were checked by flow cytometry and qPCR methods in this study. The effect of POL on TLR6 signaling pathway will be confirmed by using TLR6 knockout mice and the specific binding of POL with TLR6 protein on the DC surface will be investigated in future studies.

In conclusion, our results show for the first time that POL can activate the TLR6 signaling pathway and stimulate Th responses for HBV-specific antibody production to treat chronic HBV infection.

Materials and Methods
Animals and reagents. Female C57BL/6 and HBV-transgenic mice (aged 6–8 weeks) were purchased from the Animal Institute of the Chinese Medical Academy (Beijing, China). All mice were maintained in a specific-pathogen-free facility under a 12 h/12 h light/dark cycle, and provided with pathogen-free food and water. All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of China Agricultural University. All animal protocols (SKLAB-2016-01) were approved by the Animal Welfare Committee of China Agricultural University.

All antibodies for FACS analysis were purchased from eBioscience (San Diego, CA). POL was kindly provided by Dr. Qinghong Liu (China Agricultural University, Beijing, China). The recombinant HBV vaccine (Vac) and recombinant HBV vaccine antigen (VAg) were purchased from Beijing Tiantan Biological Products Co., Ltd (Beijing, China).

Immunization and treatment. C57BL/6 and HBV-transgenic mice were randomly divided into five groups (n = 9 per group). Mice were injected intramuscularly with 2 µg HBVsAg (VAg group), 2 µg VAg in 100 µl Recombinant HBV vaccine (including alum adjuvant, Vac group), 2 µg VAg and 1 µg POL (POL/VAg group), or 1 µg POL combined with 100 µl recombinant HBV vaccine (POL/Vac group). A control group was injected with saline. The mice were immunized on day 0 and boosted on days 14 and 28. All experiments were repeated three times. The injection sites exhibited no erythema or edema, and all mice appeared healthy after the injections.

T-cell proliferation analysis. Four days after the third immunization, three mice from each group were sacrificed and single lymphocyte suspensions were prepared from the spleen. T-cell proliferation analysis was performed using MTT staining (Sigma, St. Louis, MO) as described previously with VAg protein as a specific stimulator, anti-CD3 monoclonal antibody (mAb) as a positive control and BSA as an irrelevant antigen control. At the same time, the splenocytes were stained with CFSE (Invitrogen, Carlsbad, CA) and treated with Protein Transport Inhibitor Cocktail (eBioscience, San Diego, CA) and treated with Protein Transport Inhibitor Cocktail to stimulate cytokine production. After stimulation for 8 h, the samples were treated with Fixation/Permeabilization Diluent (eBioscience, San Diego, CA), then intracellularly immunostained with anti-mouse CD4, IFN-γ, IL-4 and IL-21 mAbs for cytokine expression analysis by flow cytometry.

Quantitative PCR. Three days after the third stimulation, total RNA from spleens of C57BL/6 and HBV-transgenic mice was prepared using a RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. Total RNA was reverse transcribed for cDNA synthesis using transcriptase (Promega, Madison, WI) and oligo(dT)18 primer (Yingweijieji trading company ltd., Shanghai, China). Quantitative PCR was performed using a LightCycler 480 System (Roche, Basel, Switzerland) and primers as follows: POL reverse, 5' TTAGCCAAAGACAGAAAAACCCCA 3'; POL6 reverse, 5' GGGACATGAGTAAGGTTCCTTTGT 3'; IL-1β
forward, 5′-TGTATGAAAGCACGGCACACC3′; IL-1β reverse, 5′-TCTTCTTTGGTATTGCTTGG3′; and for internal reference, ribosomal protein L9 (RPL9) forward, 5′-CTGAGGTCAAAGGGAATGTGTC3′; RPL9 reverse, 5′-TGTCAGCAGAAGCTTTGTTG3′. All primers were synthesized by Yingweijiel trading company ltd. (Shanghai, China). The results are presented as relative expression, normalized to RPL9. Analyses were conducted in triplicate.

**Western blot.** Three days after the third vaccination, splenocytes from immunized C57BL/6 and HBV-transgenic mice were lysed and centrifuged. The supernatants were mixed with Laemmli loading buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred onto polyvinylidene fluoride membranes (Millipore Corporation, Billerica, USA), and blotted with primary antibodies. Subsequently, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (Pierce, Appleton, WI). Antibodies against MyD88, IRAK1, TRAF6, phospho-IκB and phospho-NF-κB were purchased from Santa Cruz Biotechnology, Inc (Texas).

**Liver histology analysis.** On day seven after the third treatment, livers of HBV-transgenic mice were prepared and fixed in 4% paraformaldehyde for one week, then embedded in parafin. Serial 8-μm-thick sections were cut and affixed to slides. The slides were then deparaffinized and stained with hematoxylin and eosin for morphologic analysis. For immunohistochemistry staining, the slides were deparaffinized and stained with anti-HBVAg antibody and diaminobenzidine detection system (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd, Beijing, China). Sections were analyzed under a light microscope to detect histological changes.

**Statistical analysis.** The Student’s t-test was used to compare two groups. Analysis of variance was used for multi-group analysis. Data are expressed as means ± standard deviation. p < 0.05 was considered statistically significant.

**References**
1. Pol, S. & Michel, M.-L. Therapeutic vaccination in chronic hepatitis B virus carriers. *Expert Review of Vaccines* 5, 707–716 (2006).
2. Gamarnik, D. & Prince, A. M. Hepatitis B Virus Infection — Natural History and Clinical Consequences. *New England Journal of Medicine* 350, 1118–1129 (2004).
3. Kosinska, A. D., Zhang, E., Lu, M. & Roggendorf, M. Therapeutic Vaccination in Chronic Hepatitis B: Preclinical Studies in the Woodunch, *Hepatitis Research and Treatment* 2010, 817580 (2010).
4. De Gregorio, E., Trifon, E. & Rappuoli, R. Alum adjuvanticity: Unraveling a century old mystery. *European Journal of Immunology* 38, 2068–2071 (2008).
5. Ambrosch, F. et al. A hepatitis B vaccine formulated with a novel adjuvant system. *Vaccine* 18, 2095–2101 (2000).
6. Ioeje, U. H., Yang, H.-I. & Chen, C.-J. Natural history of chronic hepatitis B: what exactly has REVEAL Revealed? *Liver International* 32, 1333–1341 (2012).
7. Chisari, F. V. & Ferrari, C. Hepatitis B Virus Immunopathogenesis. *Annual Review of Immunology* 13, 29–60 (1995).
8. O’Hagan, D. T., Mackichan, M. L. & Singh, M. Recent developments in adjuvants for vaccines against infectious diseases. *Biomolecular Engineering* 18, 69–85 (2001).
9. Kang, Y. et al. The adjuvant effect of levamisole on killed viral vaccines. *Vaccine* 23, 5543–5550 (2005).
10. Schiller, M., Metze, D., Lugers, T. A., Grabbe, S. & Gunzer, M. Immune response modifiers – mode of action. *Experimental Dermatology* 15, 331–341 (2006).
11. Zhang, W. et al. Levamisole is a potential facilitator for the activation of Th1 responses of the subunit HBV vaccination. *Vaccine* 27, 4938–4946 (2009).
12. Fabrizi, F., Dixit, V., Messa, P. & Martin, P. Meta-analysis: levamisole improves the immune response to hepatitis B virus vaccine in dialysis patients. *Alimentary Pharmacology & Therapeutics* 32, 756–762 (2010).
13. Chou, H.-Y. et al. Hydrogel-Delivered GM-CSF Overcomes Nonresponsiveness to Hepatitis B Vaccine through the Recruitment and Activation of Dendritic Cells. *The Journal of Immunology* 185, 5468–5473 (2010).
14. Wang, X. et al. Overcoming HBV immune tolerance to eliminate HBsAg-positive hepatocytes via pre-administration of GM-CSF as a novel adjuvant for a hepatitis B vaccine in HBV transgenic mice. *Cell Mol Immunol* 13, 850–861 (2016).
15. Halperin, S. A. et al. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine* 21, 2461–2467 (2003).
16. Wang, S. et al. Heat shock protein gp96 enhances humoral and T cell responses, decreases Treg frequency and potentiates the anti-HBV activity in BALB/c and transgenic mice. *Vaccine* 29, 6348–6351 (2011).
17. Borchers, A. T., Keen, C. L. & Gershwin, M. E. Mushrooms, Tumors, and Immunity: An Update. *Experimental Biology and Medicine* 229, 393–406 (2004).
18. Yang, N. et al. Cystatin and preliminary crystallographic studies of an antitumour lectin from the edible mushroom Agrocybe aerugi. *Proteins Pept Lett* 12, 705–707 (2005).
19. Guillo, J., & Komska, G. Lectins in higher fungi. *Biochemical Systematics and Ecology* 25, 203–230 (1997).
20. Yang, N. et al. Molecular Character of the Recombinant Antitumor Lectin from the Edible Mushroom Agrocybe aerugi. *Journal of Biochemistry* 138, 145–150 (2005).
21. Pohleven, J. et al. Purification, characterization and cloning of a ricin B-like lectin from mushroom Clitocybe nebularis with antiproliferative activity against human leukemic T cells. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1790, 173–181 (2009).
22. Liu, Q., Wang, H. & Ng, T. B. Isolation and characterization of a novel lectin from the wild mushroom Xerocomus spadiceus. *Peptides* 25, 7–10 (2004).
23. Wang, H., Ng, T. B. & Ooi, V. E. C. Lectins from mushrooms. *Mycolological Research* 102, 897–906 (1998).
24. Wang, X., Liu, W. K., Ng, T. B., Ooi, V. E. C. & Chang, S. T. The immunomodulatory and antitumor activities of lectins from the mushroom Tricholoma mongolicum. *Immunopharmacology* 31, 205–211 (1996).
25. Ho, J. C. K., Sze, S. C. W., Shen, W. Z. & Liu, W. K. Mitogenic activity of edible mushroom lectins. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1671, 9–17 (2004).
26. Wang, H., Gao, J. & Ng, T. A new lectin with highly potent antitumor and antigrowth activities from the oyster mushroom Pleurotus ostreatus. *Biochem Biophys Res Commun* 275, 810–816 (2000).
27. Gao, W. et al. Mushroom lectin enhanced immunogenicity of HBV DNA vaccine in C57BL/6 and HBsAg- transgenic mice. *Vaccine* 31, 2273–2280 (2013).
28. Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T. & Sharon, N. What should be called a lectin? *Nature* 285, 66–66 (1980).
29. Underhill, D. M. Mini-review Toll-like receptors: networking for success. *European Journal of Immunology* 33, 1767–1775 (2003).
30. Bessa, J., Kopf, M. & Bachmann, M. F. Cutting Edge: IL-21 and TLR Signaling Regulate Germinal Center Responses in a B Cell- Intrinsic Manner. *The Journal of Immunology* 184, 4615–4619 (2010).
31. Sajio, S. & Iwakura, Y. Dectin-1 and Dectin-2 in innate immunity against fungi. *International Immunology* 23, 467–472 (2011).
32. Sette, A. D. et al. Overcoming T Cell Tolerance to the Hepatitis B Virus Surface Antigen in Hepatitis B Virus-Transgenic Mice. *The Journal of Immunology* 166, 1389–1397 (2001).
33. Conrad, F. & Rüdiger, H. The lectin from Pleurotus ostreatus: Purification, characterization and interaction with a phosphatase. *Phytochemistry* 36, 277–283 (1994).
34. Nurieva, R. I. et al. Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages. *Immunity* 29, 138–149 (2008).
35. Takeda, K. & Akira, S. In *Current Protocols in Immunology* (John Wiley & Sons, Inc., 2001).
36. Mancini, M., Hadchouel, M., Tiollais, P. & Michel, M.-L. Regulation of Hepatitis B Virus mRNA Expression in a Hepatitis B Surface Antigen Transgenic Mouse Model by IFN–―Secreting T Cells After DNA-Based Immunization. *The Journal of Immunology* 161, 5564–5570 (1998).
37. Zhu, D. et al. Clearing Persistent Extracellular Antigen of Hepatitis B Virus: An Immunomodulatory Strategy To Reverse Tolerance for an Effective Therapeutic Vaccination. *The Journal of Immunology* 196, 3079–3087 (2016).

Acknowledgements
This work was supported by grants from the National Natural Science Foundation of China (31470042) and Beijing Innovation for Undergraduates of China (Project: 2015bj024).

Author Contributions
Y.K. and M.H. contributed to the conception and design of this study. M.H., D.S. and W.G. carried out animal experiments. Q.H. contributed to the materials. All authors contributed to the analysis and interpretation of the data. All authors commented on the manuscript and approved the final version to be submitted.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017