Immunizations with hepatitis B viral antigens and a TLR7/8 agonist adjuvant induce antigen-specific immune responses in HBV-transgenic mice

Ying Wang[a], **Kun Chen**[a], **Zhiyuan Wu**[a], **Yuetao Liu**[b], **Shangmei Liu**[c], **Zhongmei Zou**[b], **Shu-Hsia Chen**[d], and **Chunfeng Qu**[a,*]

[a]State Key Laboratory of Molecular Oncology, Cancer Institute/Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 17 Panjiayuan Nanli, Beijing 100021, China

[b]Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

[c]Pathology Department, Cancer Institute/Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

[d]Departments of Oncological Sciences and Surgery, Mount Sinai School of Medicine, New York, New York, USA

Summary

Background—The capacity of toll-like receptor (TLR) 7/8 agonist-conjugated hepatitis B virus (HBV) proteins (HBV-Ag) to overcome established hepatitis B surface antigen (HBsAg)-specific immune tolerance was explored.

Methods—A TLR7/8 agonist, CL097, was conjugated with alum-absorbed HBsAg and hepatitis B core antigen (HBcAg), as confirmed by ultra performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF MS). Mice from two independently generated HBV-transgenic (HBV-Tg) colonies, C57BL/6J-TgN (AlblHBV) 44Bri/J mice and C57BL/6-HBV-1.3 genome-eq mice, were immunized with CL097-conjugated HBV-Ag every 2 weeks, four times.

Results—After immunization, 8/11 (72.7%) of the AlblHBV mice and 10/13 (76.9%) of the HBV-1.3 genome-eq mice generated serum detectable antibodies against HBsAg (anti-HBs). HBsAg-specific interferon gamma (IFN-γ)-producing CD4^{+} and CD8^{+} T-cells were detected in splenocytes from these mice. Naïve normal mice receiving splenocytes from the mice immunized

with CL097-conjugated HBV-Ag generated immediate recall immune responses, e.g., the mice that received CD4$^+$CD25$^+$-depleted splenocytes generated anti-HBs on day 3 after HBsAg challenge while those receiving cells from sham-immunized mice did not.

Conclusions—Immunization with CL097-conjugated HBV-Ag reversed immune tolerance in HBV-Tg mice and induced antigen-specific immune responses. TLR7/8 agonists appear to be potent adjuvants for the induction of antigen-specific Th1 responses in an immune tolerant state.

Keywords

Toll-like receptor 7/8 agonists; Antigen-specific Th1 responses; Immune tolerant state; Chronic hepatitis B virus infection

---

1. Introduction

Adjuvants are crucial for the generation of an optimal immune response to purified protein vaccines. Recent advances in our understanding of innate immunity have led to the identification of immune pathways and adjuvant formulations more suitable for clinical advancement. One area of particular interest is the discovery of agonists that target the toll-like receptors (TLRs). Signaling from the TLRs expressed on monocytes and monocyte-derived dendritic cells (moDCs), through recognition of various pathogen-associated molecular patterns, induces these cells to secrete distinct cytokines, which in turn influence T-cell differentiation.[1] Recent research has demonstrated that microbial stimulation promotes monocyte differentiation into DC-SIGN/CD209$^+$ moDCs in vivo and these moDCs exhibit a greater capacity than lymphoid resident dendritic cells (DCs) to stimulate T-cell proliferation once they acquire the antigens together with TLR4 ligands.[2] Cervarix, a prophylactic vaccine against human papillomavirus (HPV) types 16 and 18, recently received approval from the US Food and Drug Administration (FDA).[3] In this vaccine, viral antigens are formulated with monophosphoryl lipid A, a TLR4-targeted adjuvant, which confers protective immunity against HPV and promotes immune response broadening. Other adjuvants targeting TLRs are in development for new therapeutic vaccine candidates for cancers and some chronic infectious diseases.[3,4]

The idea of utilizing immunotherapy for chronic hepatitis B virus (HBV) infection is supported by findings that bone marrow transplantation of anti-HBV immunity to the recipient could cure chronic HBV infection.[5,6] A therapeutic vaccine, which represents one of the immunotherapy strategies, has been developed in different forms.[7–10] However, the clinical response to these vaccines has been poor, probably because of immune tolerance to HBV viral antigens.[11,12] Patients who recover from acute HBV infections usually have vigorous antibody responses, with antibodies against hepatitis B surface antigen (anti-HBs) easily detectable, and polyclonal T-cell responses against multiple HBV antigens (HBV-Ag).[13,14] Therefore, it is important for an effective therapeutic vaccine to induce multiple HBV antigen-specific responses by activating both antigen-specific CD4$^+$ and CD8$^+$ T-cells in the immune tolerant state.

Previously, we reported that human monocytes differentiated into moDCs when they phagocytosed dead cells containing ssRNA, the TLR7/8 agonist, and induced strong CD8$^+$

T-cell responses to the cell-associated antigens.[15] Using chemically synthesized TLR7/8 agonists we demonstrated that CL075 and CL097 stimulated newly recruited monocyte-derived cells into potent antigen-presenting cells (APCs) that enhance hepatitis B surface antigen (HBsAg) immunogenicity in both humans and mice.[16] TLR7/8 agonists conjugated to HIV Gag protein have been shown to enhance the magnitude and quality of Th1 and CD8+ T-cell responses in non-human primates.[17,18] TLR7/8 agonists appear to be good candidate adjuvants for prophylactic vaccines to induce Th1 responses in normal animals.[16–20] However, it is unknown whether TLR7/8 agonist-conjugated vaccines could break the established antigen-specific tolerance and induce antigen-specific immune responses.

2. Materials and methods

2.1. Mice and reagents

C57BL/6 male wild-type mice and two independently generated HBV transgenic (HBV-Tg) mouse colonies (males, 7–8 weeks) with C57BL/6 background were used. C57BL/6-HBV-1.3 genome-eq transgenic mice were generated in the Transgenic Laboratory, Infectious Disease Center, Guangzhou.[21] HBsAg-transgenic C57BL/ 6J-TgN (AlblHBV) 44Bri/J mice, which were originally generated in the laboratory of Dr Chisari, were purchased from Peking University, China. Both colonies constitutively express HBsAg in liver cells and secrete HBsAg in serum, as reported previously.[22] All procedures involving mice were approved by the Institutional Animal Care and Use Committee of the Cancer Institute, Chinese Academy of Medical Sciences.

Recombinant HBsAg (yeast) was from Dalian Hissen Bio-pharm Inc.; recombinant influenza A H1N1 virus M1 protein (*Escherichia coli*) was from QuantoBio Biotechnology. Endotoxin levels of these proteins were less than 1.0 EU/μg protein. Recombinant hepatitis B core antigen (HBcAg) (*E. coli*) was from Jianan Biotechnology, China (endotoxin level 0.14 EU/μg protein). ELISA kits for quantifying HBsAg and anti-HBs were purchased from Wantai Biological Pharmacy, China. The TLR7/8 agonist, CL097, was purchased from InvivoGen (CA, USA); aluminum hydroxide gel was from Sigma-Aldrich (MO, USA). All chemicals for ultra performance liquid chromatography (UPLC) were analytical grade.

Detailed information on the materials and methods are provided in the Supplementary Material.

2.2. Preparation of vaccines and immunization protocol

To prepare the TLR7/8 agonist-conjugated vaccine, HBsAg and HBcAg (5 μg of each) were diluted in 50 μl normal saline and mixed with the same volume of aluminum hydroxide (500 μg) at room temperature; 5 μg CL097 was then added. For immunization, each mouse received 5 μg HBV-Ag every 2 weeks.

2.3. Confirmation of CL097 conjugation with alum-absorbed HBV-Ag by UPLC-Q/TOF MS

UPLC quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF MS) was performed using an Acquity UPLC BEH C18 column on the Acquity UPLC system, equipped with

SYNAPT G2 HDMS (Waters Corp., UK) with an electrospray ionization source (ESI) in positive ion mode. The column was maintained at 40 °C and eluted at a flow rate of 0.45 ml/min, using a mobile phase of 0.1% formic acid in water (18.2 mΩ) and acetonitrile (HPLC grade; J.T. Baker, Phillipsburg, NJ, USA). Leucine-enkephalin was used as the lock mass in all analyses (*m/z* 556.2771) at 0.5 μg/ml, with a flow rate of 5 ml/min. Data were collected in centroid mode from *m/z* 100 to *m/z* 1500.

A series of standard working solutions was prepared and 5 μl of each was injected into the UPLC system for analysis after centrifugation at 6500 *g* for 5 min. CL097-conjugated HBV-Ag solution was divided into two parts after the same centrifugation. The pellet was resuspended in 20% sodium citrate solution and incubated at 37 °C for 2 h followed by the same centrifugation. All the supernatant was analyzed by UPLC-Q/TOF MS.

2.4. Flow cytometry analysis (FACS; fluorescence activated cell sorting)

Fluorescence-conjugated antibodies to mouse CD3, CD4, CD8, interferon gamma (IFN-γ), CD25, and FoxP3 were purchased from eBioscience (CA, USA). Flow cytometry staining for cell surface markers or intracellular cytokines was done using standard laboratory protocols. Data were acquired in an LSR-II (Becton-Dickinson, San Diego, CA) and analyzed using Flowjo software (Tree Star Inc, Asland, OR).

2.5. Assays for HBsAg-specific antibody-secreting cells (ASCs)

The presence of HBsAg-specific ASCs in immunized mouse splenocytes was determined by ELISPOT assay, as described in the literature.[23] Further details of the materials and methods are provided in the Supplementary Material.

2.6. Assays for HBsAg-specific T-cell cytokine production

Splenocytes were isolated from immunized normal or HBV-Tg mice and cultured in the presence of 5 mg HBsAg for 72 h, followed by the addition of 1 × Brefeldin A Solution (Becton-Dickinson, San Diego, CA) for another 6 h. Cells were collected and stained using standard FACS staining protocols.

2.7. Cell sorting and transfer

Single splenocytes of HBV-1.3 genome-eq transgenic mice that were untreated or immunized with CL097-conjugated HBV-Ag, were labeled with fluorescein isothiocyanate (FITC)-conjugated CD4 and PE-Cy5-conjugated CD25 antibodies. The cells were sorted into two parts: CD4$^+$CD25$^+$ (Treg cells) and CD4$^+$CD25$^+$-depleted populations (non-Treg) using a FACSAria (BD, USA). Because Treg is only about 10% of peripheral CD4$^+$ T-cells,[24] each Naïve C57BL/6J mouse received 2×10^5 Treg or 2×10^6 non-Treg cells from sham-treated or immunized mice (in 200 μl phosphate buffered saline) via the tail vein.[25] On the following day, all recipient mice were challenged intramuscularly with 5μg recombinant HBsAg or 5 μg recombinant influenza A H1N1 M1 proteins. Serum samples were collected on days 3, 6, 9, and 12. Anti-HBs serum levels were quantified using a commercialized ELISA kit; anti-M1 levels were determined using a direct ELISA assay developed in our laboratory.

2.8. Statistical analysis

The statistical analysis was conducted using SPSS software (11.0). Statistical tests were performed using all-pairs Tukey–Kramer analysis and/or the two-tailed Student's *t*-test.

3. Results

3.1. Binding of TLR7/8 agonist with particulate antigens is necessary to induce optimal humoral and cellular immune responses against HBsAg

Under physiological conditions (pH 7.2–7.4), alum hydroxide adjuvants have a positive surface charge, while CL097, HBsAg, and HBcAg have a negative surface charge. Theoretically, CL097 binds to alum via the surface charge using the bond present in the N atom (Figure 1A). Using UPLC-Q/TOF MS, we confirmed the conjugation of CL097 to alum (Figure 1B). The conjugation was stable at normal pH but was partially dissociated when sodium citrate solution was added (Table 1). Conjugation of HBV-Ag, the HBsAg and HBcAg proteins, with alum was confirmed using the bicinchonnic acid (BCA) assay (Table 1).

In comparison to the immunized mice with alum absorbed HBV-Ag (G1 in Figure 1C), the serum level of anti-HBs in the immunized mice using CL097-conjugated HBV-Ag through alum was significantly increased (G2, in Figure 1C). The antibodies against HBcAg in the two groups of mice showed a similar pattern (data not shown). However, no similar effect on antibody production was observed when CL097 was injected in free form, without being absorbed with HBV-Ag through alum (G3 in Figure 1C), or mixed with HBV-Ag without alum (G4 in Figure 1C). The percentage of HBsAg-specific, HBcAg-specific IFN-γ-producing T-cells was also significantly increased in the mice immunized with CL097-conjugated HBV-Ag (with CL097, in Figure 1D; Supplementary Material, Figure S1) compared to the mice immunized with HBV-Ag alone (without CL097, in Figure 1D; Supplementary Material, Figure S1).

3.2. Immunization with CL097-conjugated HBV-Ag results in the generation of anti-HBs antibodies and HBsAg-specific T-cells in HBV-Tg mice

The effects of TLR7/8 agonist-conjugated HBV-Ag were then examined in HBV-Tg mice. To confirm the results, we tested it in two independently prepared HBV-Tg mouse colonies. In the AlblHBV 44Bri/J mice, a total of 8/11 (72.7%) generated serum anti-HBs antibodies following immunization with four doses of CL097-conjugated HBV-Ag, averaging 636 ± 258 mIU/ml (Figure 2A). In the C57BL/6-HBV-1.3 genome-eq mice, a total of 10/13 (76.9%) of the mice had serum detectable anti-HBs antibodies, with an average of 425 ± 118 mIU/ml (Figure 2B).

The effect of the serum HBsAg concentration on anti-HBs production was analyzed in the C57BL/6-HBV-1.3 genome-eq mice. Generally, no correlation was found between serum anti-HBs levels and the corresponding pre-immunized serum HBsAg concentration in each mouse ($R^2 = 0.077$, $p = 0.357$) (Figure 2C). However, anti-HBs was detectable in all mice with less than 3 μg/ml serum HBsAg (Figure 2C).

Anti-HBs persisted for more than 20 weeks after antibodies were initially induced. Following a single dose of vaccine booster, the concentration of anti-HBs increased dramatically (Figure 2D). Mice treated with alum-conjugated CL097 alone (sham-immunization) had no detectable serum anti-HBs (Figure 2, A and B). Serum alanine aminotransferase (ALT) levels were elevated in some vaccine-immunized and some sham-immunized HBV-Tg mice. On weeks 10 and 20, serum ALT returned to the same levels as untreated HBV-Tg mice (Figure 2E).

Representative mice that had anti-HBs generated were euthanized at week 22 after the vaccine booster. Splenocytes from the mice immunized with CL097-conjugated HBV-Ag contained more HBsAg-specific B-cells than those from sham-immunized mice (Figure 3, A and B). After the splenocytes were stimulated with HBsAg in vitro, IFN-γ-producing CD4$^+$ and CD8$^+$ T-cells were also detected in immunized HBV-Tg mice with a more obvious increase in the number of IFN-γ-producing HBsAg-specific CD8$^+$ T-cells compared to the sham-immunized mice (Figure 3C). These results indicate that inclusion of TLR7/8 agonist CL097 in the alum-absorbed vaccine results in induction of a robust immune response against tolerant HBsAg antigens in HBV-Tg mice.

3.3. Immunization with CL097-conjugated HBV-Ag breaks antigen-specific immune tolerance in HBV-Tg mice

CD4$^+$CD25$^+$ Treg cells play an important role in maintaining immune tolerance. The percentage of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells decreased significantly in the vaccine-immunized (2.6%) versus sham-immunized mice (6.72%) (Figure 4A). We then sorted splenocytes from the immunized mice into two populations – CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$-depleted cells – and transferred each of them intravenously into naïve C57BL/6J mice, followed by challenge with HBsAg or non-relevant influenza A virus M1protein (depicted in Figure 4B). The mice that received CD4$^+$CD25$^+$-depleted splenocytes from immunized mice generated anti-HBs immediately after HBsAg challenge, while those receiving cells from sham-immunized mice did not. Antibodies were detected as early as on day 3 and reached a plateau by day 9 (Figure 4C). The mice that received CD4$^+$CD25$^+$ cells from the vaccine-immunized mice generated anti-HBs antibodies by day 12 (Figure 4C). Mice receiving splenocytes from vaccine- or sham-immunized HBV-Tg exhibited similar responses to non-relevant M1 proteins (Figure 4D). These results suggest that immune tolerance to HBsAg was broken and specific immune responses against HBsAg were generated in HBV-Tg mice by immunization with CL097-conjugated HBV-Ag.

4. Discussion

Most chronic HBsAg carriers are infected in early life,[26] and viral persistence has been associated with a defect in the development of specific immunity against HBV viral proteins.[5,6,27] It is important for therapeutic vaccines to overcome immune tolerance and induce both specific antibodies against HBsAg and T-cell immunity against HBV structural proteins in the presence of chronic HBV infection.[11,12,27] Our results demonstrated that binding of TLR7/8 agonist to alum-absorbed HBV-Ag was able to break the immune tolerance and induce humoral and cellular immune responses against HBsAg in HBV-Tg mice.

Many TLR agonists can be potent adjuvant candidates for improving the magnitude and quality of memory T-cell responses.[4,17,18] However, systemic activation of DCs by TLR ligands inhibits their antigen presentation function.[28] Monocytes express a variety of TLRs, patrol various tissues for signs of infection and inflammation, and are approximately 20 times more abundant than DCs in blood and bone marrow. Therefore, monocytes are a promising target for the design of prophylactic and therapeutic vaccines against some chronic diseases.[2,29] TLR7/8 are mainly expressed in intracellular compartments and sense the ligands when the cells take up the ligands.[30] In this study, we demonstrated that binding CL097 with HBV-Ag through alum resulted in better immune responses than did the free form of CL097. We reported previously that the immunogens were mainly taken up by monocytes and monocyte-derived cells after immunization.[16] Therefore, TLR7/8 conjugation with immunogens appeared important for inducing monocyte differentiation into potent moDCs that could prime naïve T-cells.

TLR expression patterns are different in mice and humans. Distinct DC subsets also have different TLR expression patterns.[1] TLR7 and TLR8 are intracellularly expressed and recognize ssRNA and synthetic antiviral imidazoquinoline components.[1,30] In humans, TLR7 is mainly expressed in plasmacytoid DCs (pDCs), which produce high levels of type I IFNs in response to microbial stimulation. TLR8 is expressed in various tissues, with the highest levels in myeloid DCs and monocytes.[1,31] In mice, TLR8 is nonfunctional and TLR7/8 agonists work through TLR7, which is expressed in murine monocytes and moDCs. The effect in humans might be different from what we observed in this murine system. However, stimulation with TLR7/8 agonists in both humans and mice has been shown to generate Th1-polarizing moDCs.[16,20,32]

An elevation of serum ALT levels was observed in our vaccine-immunized mice, which might be harmful for patients with chronic HBV infection. Clinically, patients exhibit increased serum ALT levels during and after seroconversion from the status of hepatitis e antigen (HBeAg)-positive to anti-HBe-positive as a result of the immunological elimination of HBV-expressing liver cells. Consequently, this translates into reduced viral loads and clinical remission.[33] In this study, HBsAg-specific B-cells and IFN-γ-producing CD4$^+$ and CD8$^+$ T-cells were only detected in vaccine-immunized but not in sham-treated HBV-Tg mice. The elevated serum ALT levels in vaccine-immunized mice were probably due to the effect of immunological elimination on HBsAg-expressing liver cells. However, we could not exclude the possibility of non-specific damage caused by the TLR7/8 agonists on liver cells, since the sham-treated mice also exhibited elevated ALT.

In conclusion, HBV-Ag conjugated with TLR7/8 agonists reversed the HBsAg-specific non-responses in HBV-Tg mice. TLR7/8 agonists appear to be good candidate adjuvants for therapeutic vaccines to induce Th1 responses in the presence of a tolerant state, in addition to their reported effects when used in prophylactic vaccines.[16–20]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011; 34:637–50. [PubMed: 21616434]

2. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, et al. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. Cell. 2010; 143:416–29. [PubMed: 21029863]

3. Dubensky TW Jr, Reed SG. Adjuvants for cancer vaccines. Semin Immunol. 2010; 22:155–61. [PubMed: 20488726]

4. Mbow ML, De Gregorio E, Valiante NM, Rappuoli R. New adjuvants for human vaccines. Curr Opin Immunol. 2010; 22:411–6. [PubMed: 20466528]

5. Ilan Y, Nagler A, Adler R, Naparstek E, Or R, Slavin S, et al. Adoptive transfer of immunity to hepatitis B virus after T cell-depleted allogeneic bone marrow transplantation. Hepatology. 1993; 18:246–52. [PubMed: 8340054]

6. Lau GK, Suri D, LiangR, Rigopoulou EI, Thomas MG, Mullerova I, et al. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. Gastroenterology. 2002; 122:614–24. [PubMed: 11874993]

7. Mancini M, Hadchouel M, Davis HL, Whalen RG, Tiollais P, Michel ML. DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. Proc Natl Acad Sci U S A. 1996; 93:12496–501. [PubMed: 8901610]

8. Hong Y, Peng Y, Mi M, Xiao H, Munn DH, Wang GQ, et al. Lentivector expressing HBsAg and immunoglobulin Fc fusion antigen induces potent immune responses and results in seroconversion in HBsAg transgenic mice. Vaccine. 2011; 29:3909–16. [PubMed: 21421003]

9. Niedre-Otomere B, Bogdanova A, Skrastina D, Zajakina A, Bruvere R, Ose V, et al. Recombinant Semliki Forest virus vectors encoding hepatitis B virus small surface and pre-S1 antigens induce broadly reactive neutralizing antibodies. J Viral Hepat. 2012; 19:664–73. [PubMed: 22863271]

10. Akbar SM, Furukawa S, Horiike N, Abe M, Hiasa Y, Onji M. Safety and immunogenicity of hepatitis B surface antigen-pulsed dendritic cells in patients with chronic hepatitis B. J Viral Hepat. 2011; 18:408–14. [PubMed: 20487261]

11. Michel ML, Deng Q, Mancini-Bourgine M. Therapeutic vaccines and immune-based therapies for the treatment of chronic hepatitis B: perspectives and challenges. J Hepatol. 2011; 54:1286–96. [PubMed: 21238516]

12. Xu DZ, Wang XY, Shen XL, Gong GZ, Ren H, Guo LM, et al. Results of a phase III clinical trial with an HBsAg–HBIG immunogenic complex therapeutic vaccine for chronic hepatitis B patients: experiences and findings. J Hepatol. 2013; 59:450–6. [PubMed: 23669281]

13. Maini MK, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med. 2000; 191:1269–80. [PubMed: 10770795]

14. Yang PL, Althage A, Chung J, Maier H, Wieland S, Isogawa M, et al. Immune effectors required for hepatitis B virus clearance. Proc Natl Acad Sci U S A. 2010; 107:798–802. [PubMed: 20080755]

15. Qu C, Nguyen V, Merad M, Randolph G. MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells. J Immunol. 2009; 182:3650–9. [PubMed: 19265143]

16. Du J, Wu Z, Ren S, Wei Y, Gao M, Randolph GJ, et al. TLR8 agonists stimulate newly recruited monocyte-derived cells into potent APCs that enhance HBsAg immunogenicity. Vaccine. 2010; 28:6273–81. [PubMed: 20637759]

17. Wille-Reece U, Flynn BJ, Lore K, Koup RA, Kedl RM, Mattapallil JJ, et al. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. Proc Natl Acad Sci U S A. 2005; 102:15190–4. [PubMed: 16219698]

18. Wille-Reece U, Flynn BJ, Lore K, Koup RA, Miles AP, Saul A, et al. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. J Exp Med. 2006; 203:1249–58. [PubMed: 16636134]

19. Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood. 2006; 108:1284–90. [PubMed: 16638933]

20. Kastenmuller K, Wille-Reece U, Lindsay RW, Trager LR, Darrah PA, Flynn BJ, et al. Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. J Clin Invest. 2011; 121:1782–96. [PubMed: 21540549]

21. Liu G, Xiong Y, Wang H, Jia Y, Zhou J, Pan F, et al. Establishment of high level expression HBV transgenic BALB/c mice and its genetic and expressive stability. Chinese Journal of Veterinary Science. 2003; 23:580–2.

22. Chisari FV, Pinkert CA, Milich DR, Filippi P, McLachlan A, Palmiter RD, et al. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. Science. 1985; 230:1157–60. [PubMed: 3865369]

23. Shen M, Wang S, Ge G, Xing Y, Ma X, Huang Z, et al. Profiles of B and T cell immune responses elicited by different forms of the hepatitis B virus surface antigen. Vaccine. 2010; 28:7288–96. [PubMed: 20831917]

24. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. Nat Rev Immunol. 2012; 12:157–67. [PubMed: 22322317]

25. Sharma MD, Hou DY, Baban B, Koni PA, He Y, Chandler PR, et al. Reprogrammed foxp3(+) regulatory T cells provide essential help to support cross-presentation and CD8(+) T cell priming in naive mice. Immunity. 2010; 33:942–54. [PubMed: 21145762]

26. Edmunds WJ, Medley GF, Nokes DJ, Hall AJ, Whittle HC. The influence of age on the development of the hepatitis B carrier state. Proc Biol Sci. 1993; 253:197–201. [PubMed: 8397416]

27. Chisari FV, Isogawa M, Wieland SF. Pathogenesis of hepatitis B virus infection. Pathol Biol (Paris). 2010; 58:258–66. [PubMed: 20116937]

28. Wilson NS, Behrens GM, Lundie RJ, Smith CM, Waithman J, Young L, et al. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. Nat Immunol. 2006; 7:165–72. [PubMed: 16415871]

29. Qu C, Brinck-Jensen NS, Zang M, Chen K. Monocyte-derived dendritic cells: targets as potent antigen-presenting cells for the design of vaccines against infectious diseases. Int J Infect Dis. 2014; 19:1–5. [PubMed: 24216295]

30. Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity. 2010; 32:305–15. [PubMed: 20346772]

31. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol. 2002; 3:499. [PubMed: 12032557]

32. Spranger S, Javorovic M, Burdek M, Wilde S, Mosetter B, Tippmer S, et al. Generation of Th1-polarizing dendritic cells using the TLR7/8 agonist CL075. J Immunol. 2010; 185:738–47. [PubMed: 20511554]

33. Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, et al. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology. 2002; 35:1522–7. [PubMed: 12029639]
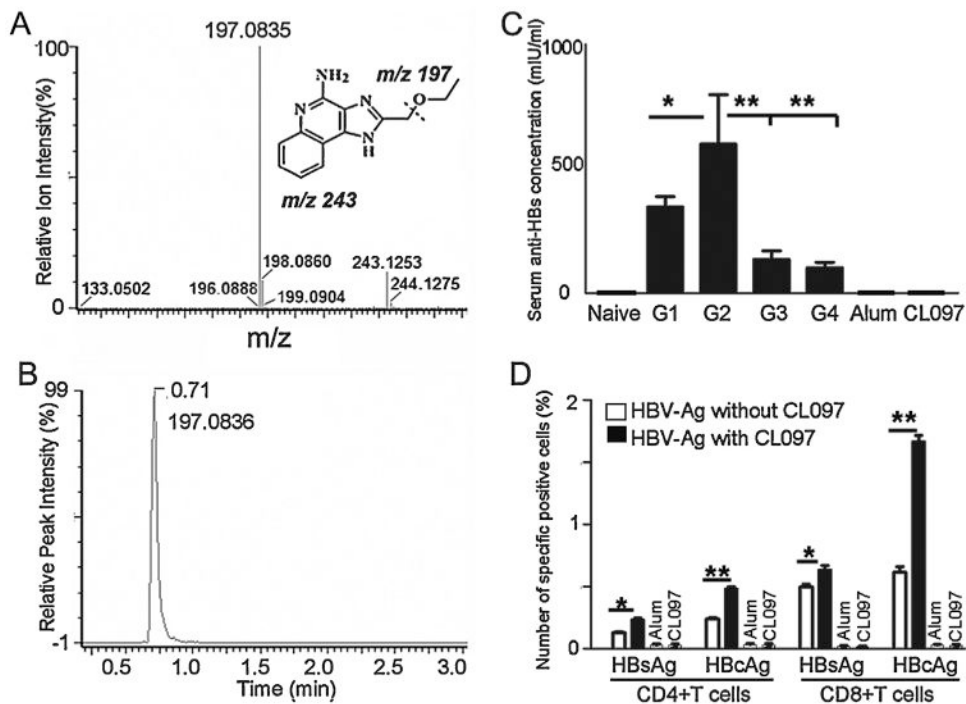
Figure 1.
Effectof TLR7/8 agonist on specific immune responses against HBsAg in wild-type
C57BL/6 mice. (A) Structure of CL097 and its ESI mass spectrum (m/z 243→197) in
positive ion mode performed in UPLC-Q/TOF MS. (B) Relative peak intensity of CL097
bound with alum. (C) Serum levels of anti-HBs measured 2 weeks after the second
immunization (five mice per group, each was repeated three times). G1: immunization with
5 μg HBV-Ag absorbed to alum; G2: immunization with 5 μg HBV-Ag and 5 μg CL097
absorbed to alum; G3: immunization as for G1 in one side of the mice, 5 μg CL097 solution
was injected in the other side; G4: immunization with 5 μg HBV-Ag and 5 μg CL097
without absorbing to alum. No anti-HBs was detectable in the naïve mice or the mice
immunized with aluminum hydroxide alone (Alum) or CL097 alone (CL097); *$p < 0.05$,
**$p < 0.01$ determined by all-pairs Tukey–Kramer analysis. (D) Frequency of HBsAg-
specific, HBcAg-specific CD4^{+} and CD8^{+} IFN-γ-producing T-cells in the group pooled
splenocytes based on FACS staining (Supplementary Material, Figure S1) (five mice per
group, each was repeated three times); *$p < 0.05$, **$p < 0.01$ determined by two-tailed
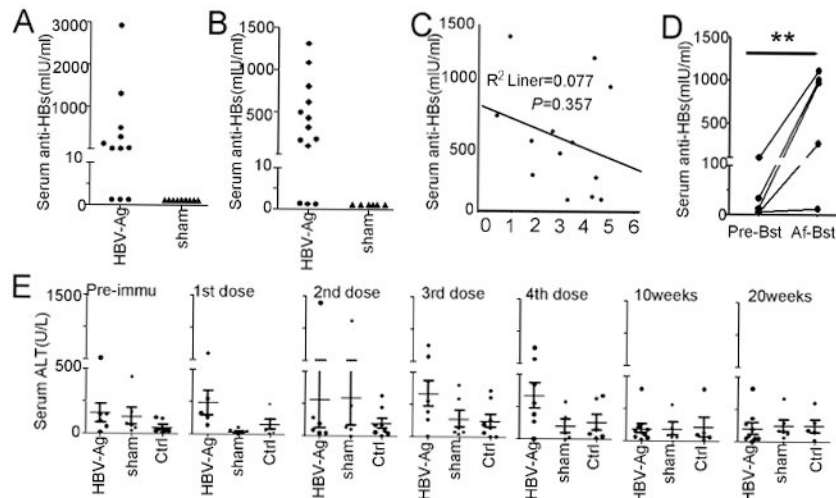Student's t-test.

Figure 2.
Humoral immune responses to HBsAg in HBV-Tg mice immunized with CL097-conjugated HBV-Ag. (A,B) Serum anti-HBs levels measured at 2weeks after four-dose immunization with CL097-conjugated HBV-Ag (HBV-Ag) in Alb1HBV 44Bri/J mice (A, $n = 11$) and in HBV-1.3 genome-eq mice (B, $n = 13$). Alum absorbed CL097 was used as the sham-immunization (sham). Each dot represents one mouse. (C) Spearman's correlation analysis between serum anti-HBs levels and the pre-immunized serum concentration of HBsAgin individual HBV-1.3 genome-eq mice. (D) Serum concentration of anti-HBs measured 20 weeks after the fourth immunization (Pre-Bst) and 2 weeks after one dose of booster (Af-Bst) using CL097-conjugated HBV-Ag ($n = 5$); $**p < 0.01$ determined by two-tailed Student's t-test. (E) Serum ALT levels measured at different time points before (pre-immu) and after the immunization. Each dot represents one mouse.
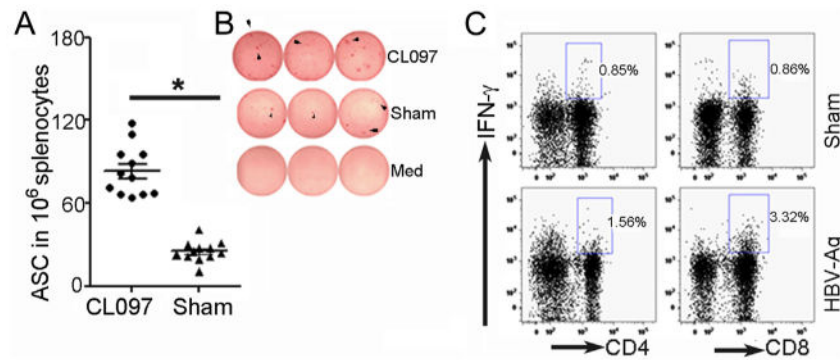
Figure 3.
HBsAg-specific B-cells and T-cells in immunized HBV-Tg mice. (A) The numbers of HBsAg-specific antibody secreting cells (ASC) were determined by ELISPOT assay 2 weeks after boosting with CL097-conjugated HBV-Ag (CL097). Samples from the mice immunized with alum absorbed CL097 alone (sham) were also assayed. Wells not coated with HBsAg were used as spontaneous release controls (Med); *$p < 0.05$ determined by two-tailed Student's *t*-test. (B) Images showing representative ASC spots in each sample well. Each spot (black arrows) represents one HBsAg-specific B-cell. (C) Splenocytes isolated from mice immunized with CL097-conjugated HBV-Ag (HBV-Ag) or the mice immunized with alum absorbed CL097 alone (Sham) were re-stimulated with HBsAg. HBsAg-specific IFN-γ-producing T-cells were determined by intracellular cytokine staining (representative of five independent experiments).
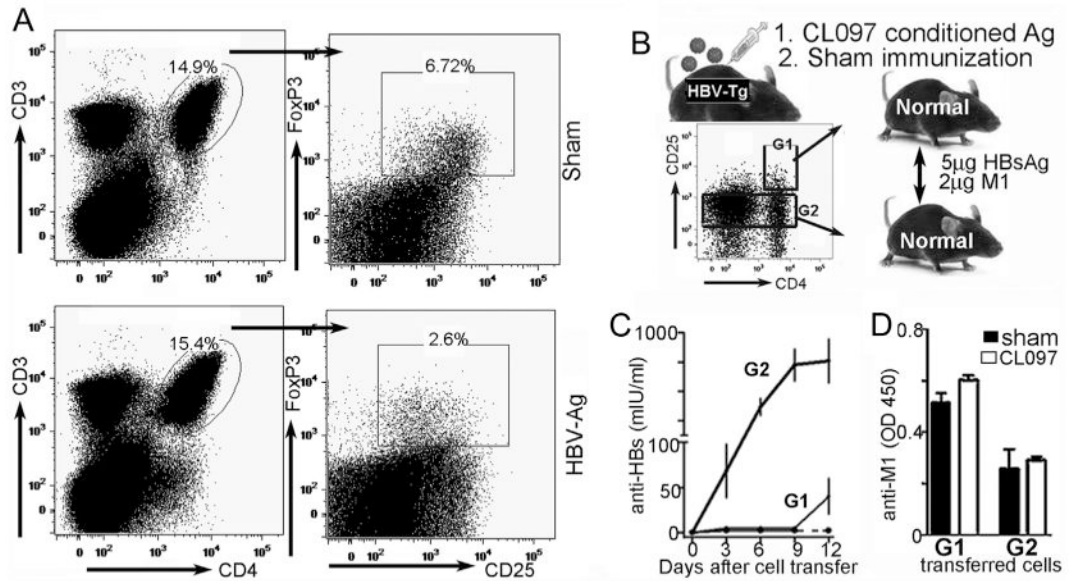
Figure 4.
Frequency and function of Treg cells in the mice immunized with CL097-conjugated HBV-Ag. (A) Frequency of CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$Treg cells in splenocytes of the sham-immunized (sham) or the mice immunized with CL097-conjugated HBV-Ag (CL097) (representative of three independent experiments). (B) Sorting strategy of CD4$^+$CD25$^+$ Treg (G1) and CD4$^+$CD25$^+$-depleted (G2) cell populations. Each of the sorted cell populations, 2 × 10^5 cells/mouse for CD4$^+$CD25$^+$ and 2 × 10^6 cells/mouse for CD4$^+$CD25$^+$-depleted cells, were injected intravenously into Naïve C57BL/6 mice (n = 3). Dot plot indicating the splenocytes from mice immunized with CL097-conjugated HBV-Ag. On the day of cell transfer, each mouse received 5 µg HBsAgor 5 µg influenza A virus M1 protein intramuscularly. (C) Anti-HBs in mice that received the G1 or G2 cells, as indicated in Figure 4B, from sham-immunized mice was undetectable (dashed line). Serum anti-HBs levels in mice that received cells from CL097-conjugated HBV-Ag immunized mice are shown as solid lines. (D) Serum levels of antibodies against influenza A virus M1 proteins in the same mice on day 12.

Table 1

Binding of CL097 and HBV antigens with different amounts of aluminum hydroxide

Aluminum hydroxide (µg)	Added CL 097 (5 µg)				Added HBV antigens (10 µg)			
	Normal saline		20% sodium citrate		Normal solution		20% sodium citrate	
	Bound	Free	Bound	Free	Bound	Free	Bound	Free
0	0	4.98	0	4.98	0	10.8	0	10
200	4.23	0.75	2.56	2.42	8.8	0	0	8.8
500	4.95	0.03	2.05	2.93	10	0	0	10
750	4.73	0.25	2.04	2.94	10.1	0	0	10
1000	4.93	0.05	2.78	2.2	10.2	0	0	10.2

HBV, hepatitis B virus.