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Induction of humoral and cellular immune response to HBV vaccine can be up-regulated by STING ligand

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ABSTRACT

A persistent hepatitis B virus (HBV) infection is characterized by a lack of or a weak immune response to HBV. Efficient induction of the HBV-specific immune response leads to the clearance of HBV. Stimulator of interferon (IFN) genes (STING) is a cytoplasmic sensor of intracellular DNA from microbes and host cells. In the present study, we examined the efficacy of cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) that is a ligand of the STING pathway as an HBV vaccine adjuvant. Wild-type (WT) mice and HBV-transgenic (HBV-Tg) mice were immunized with hepatitis B surface antigen (HBsAg) and cGAMP. The vaccination with HBsAg and cGAMP significantly enhanced the humoral and cellular immune response to HBsAg in WT and HBV-Tg mice. Cytokine production related to Th1 and Th2 responses and the activation of antigen-presenting cells in lymphoid tissues were induced by cGAMP. Vaccination using cGAMP may overcome tolerance in patients with chronic HBV infection.

1. Introduction

Infection with hepatitis B virus (HBV) is one of the most common serious infectious diseases worldwide. Approximately 2 billion people have been exposed to HBV, and 3–5% of these people have chronic HBV infection (Michel, 2002). Chronic HBV carriers can develop severe liver diseases, such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The HBV-related liver diseases cause an estimated 500,000–1,000,000 deaths each year in the world. HBV vaccines based on recombinant HBV surface antigen (HBsAg) are used for preventing HBV infection and transmission. Since the immunogenicity of HBsAg is not strong, aluminum hydroxide is used as a typical adjuvant for HBV vaccination (Michel, 2002). However, aluminum-based HBV vaccination decreases the efficacy in elderly people and is not effective in a therapeutic situation (Tohme et al., 2011). Therefore, the efficacies of many adjuvants such as Toll-like receptor agonists have been examined in HBV vaccination (Chuai et al., 2013; Li et al., 2015).

Stimulator of interferon (IFN) genes (STING), which is located on the endoplasmic reticulum, is a cytoplasmic sensor of intracellular DNA from such as microbes and host cells (Li and Chen, 2018). STING stimulated by fragmented DNA activates the TBK1-IRF3 pathway. Activated IRF3 induces type I interferon (IFN) and triggers host immune system activation. STING ligands have an anti-tumor effect in mouse cancer models (Li et al., 2016; Ohkuri et al., 2017). Cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP-AMP, cGAMP) is a ligand of the STING pathway that induces the production of type I IFN. The treatment with cGAMP activates dendritic cells (DCs) and enhances cross-presentation of tumor-associated antigens to CD8 T cells (Junkins et al., 2018; Skrnjug et al., 2014). A therapeutic vaccination for chronic HBV infection must activate DCs and induce a HBV-specific T cell response to HBV antigen.

In the present study, we examined the effect of cGAMP on the induction of a humoral and cellular HBV-specific immune response in wild type mice and HBV transgenic (Tg) mice. We show that cGAMP administration strongly induced an HBV-specific immune response via up-regulation of cytokines and chemokines that are required for the induction of optimal immune response.

2. Methods

2.1. Mice

WT male and female B10.D2 (H-2d) mice (age 8–10 weeks; weight 25–30 g) were obtained from Japan SLC Inc. (Shizuoka, Japan). HBsAg transgenic mice lineage 107–5D (official designation Tg [Alb-1, HBV] Bri66; inbred B10.D2, H-2d) in which the HBV envelope coding region

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was under the control of the mouse albumin promoter were generously provided by Dr. Francis V. Chisari (Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the guidelines for the care and use of animals established by the Animal Care and Use Committee of Gifu University, Japan.

2.2. Reagents

Recombinant HBsAg (r-HBsAg) that did not contain aluminum was obtained from Meridian Life Science, Inc. (Memphis, TN). Cyclic GMP-AMP (cGAMP) was purchased from InvivoGen (San Diego, CA). The HBsAg peptides IPQSLDSWWTSL, which bound to MHC class I molecules, and FFLLTRILTIPQSLD, which bound to MHC class II molecules, were synthesized at KURABO (Osaka, Japan).

2.3. Detection of specific antibodies against HBsAg

The serum samples were obtained from immunized mice. The concentration of hepatitis B surface antibody (HBsAb) in the serum was measured by an automated analyzer, HISCL-5000 (Sysmex corporation, Kobe, Japan).

2.4. ELISPOT assay

The antigen-specific cellular immune response was assessed by ELISPOT assay as described previously (Ito et al., 2015a, 2015b, 2017). Mice were subcutaneously inoculated with HBsAg (3 μg/mouse), HBsAg (3 μg/mouse) + cGAMP (2 μg/mouse), or HBsAg (3 μg/mouse) + CpG oligonucleotide (CpG ODN) (10 μg/mouse) 0 days and 7 days in the end of the tail. Single-cell suspensions were prepared from the whole spleen and the intrahepatic lymphocytes (IHLs) 14 days after the first immunization. A total of 3.0×10^5 splenocytes/well were stimulated for 18 h with 0–5 μg/ml of HBsAg peptide IPQSLDSWWTSL in 96 well MultiScreen filter plates (Millipore,Billerica, MA) pre-coated with a monoclonal rat anti-interferonγ (IFN-γ) antibody (R4–6A2, BD Biosciences, Franklin Lakes, NJ). The plates were washed and then incubated with a biotinylated polyclonal goat anti-IFN-γ antibody (R&D Systems, Minneapolis, MN) and then streptavidin-alkaline phosphatase. Spots were visualized by the addition of a 5-bromo-4-chloro-3-indolyl phosphatase solution (Sigma-Aldrich, St. Louis, MO) and counted manually under a microscope (40 ×magnification). The number of cytokine-secreting cells was determined by a single blinded observer, and all data were generated by analyzing three separate wells per sample.

2.5. Flow cytometric analysis of splenocytes

Splenocytes and IHLs were isolated from the immunized mice as described (Ito et al., 2003). Cell viability and cell number were assessed using a trypan blue exclusion assay. For flow cytometry, 2×10^5 splenocytes were stained with labeled antibodies using a standard protocol. The following antibodies were used: PE- or APC-labeled CD4 monoclonal antibody (mAb) (clone GK1.5; Biolegend); PE- or FITC-labeled anti-mouse CD8 mAb (clone 53–6.7; Biolegend); PE-labeled anti-mouse CD11a mAb (clone M17/4; Biolegend); PE-Cy7-labeled anti-mouse CD49d mAb (clone M17/4; Biolegend); APC-Cy7-labeled anti-mouse TNF-α mAb (clone MIP6-XT22; Biolegend); APC-labeled anti-mouse IL-2 mAb (clone JES6-5H4; Biolegend); FITC-labeled anti-mouse IFN-γ mAb (clone XMG1.2; BD Biosciences); VioBlue-labeled anti-mouse CD11c mAb (clone N418; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), FITC-labeled anti-mouse CD86 mAb (clone 24F; BD Biosciences); PE-labeled anti-mouse CD40 mAb (clone FGK45.5; Miltenyi Biotec); APC-labeled anti-mouse MHC-II mAb (clone M5/114.15.2); eBioscience);and PE-Cy7-labeled anti-mouse CD69 mAb (clone H1.2F3; eBioscience). Samples were acquired on a flow cytometer and data analysis was performed using FACSDiva software (BD Biosciences).

2.6. Real-time reverse transcription-PCR

Total RNA was isolated and transcribed into complementary DNA (cDNA) using an RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) and a high capacity cDNA transcription kit (Applied Biosystems, Foster City, CA). The resulting cDNA was used as a template for real-time PCR along with primer-probe sets for IFN-β, IFN-γ, IL-2, IL-4, IL-12b, IL-17a, TNF-α, CCL2, CXCL10, and 18S rRNA (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA) and TaqMan universal PCR master mix (Applied Biosystems) according to the manufacturer's instructions (Applied Biosystems). 18S rRNA was used as an internal control. Real-time PCR was carried out using a Light-Cycler 480 system (Roche Diagnostic Systems, Switzerland).

2.7. Intracellular cytokine staining

For intracellular staining, the splenocytes from the mice administered HBsAg or HBsAg + cGAMP were incubated for 4 h with brefeldin A (1 μg ml) and HBsAg peptide IPQSLDSWWTSL. Then, cells were fixed and permeabilized with the Cytofix/Cytoperm buffer (BD Biosciences) and stained with APC-Cy7-labeled anti-mouse TNF-α mAb, APC-labeled anti-mouse IL-2 mAb, and FITC-labeled anti-mouse IFN-γ mAb. Samples were acquired on a flow cytometer and data analysis was performed using FACSDiva software (BD Biosciences).

2.8. Histological examination

Histopathological examination of the liver was performed 7 days after the second immunization. The liver tissues were fixed in phosphate-buffered 10% formalin in PBS for 48 h and embedded in paraffin. Tissue sections were cut at 2 μm, deparaffinized, stained with H&E, and examined under light microscopy.

2.9. Statistics

Values were calculated as the mean ± SEM. Differences between experimental and control groups were analyzed using the Kruskal-Wallis test followed by Scheffe's F-test. Significance was assumed at $P < 0.05$.

3. Results

3.1. cGAMP enhances the humoral and cellular immune response in HBsAg vaccination

The ability of cGAMP to induce HBsAb was examined after the one or two immunizations with recombinant-HBsAg (r-HBsAg) and cGAMP in WT mice. We also compared cGAMP and CpG ODN in their abilities as adjuvant because CpG ODN up-regulates the cellular and humoral immune response in HBV vaccination. cGAMP significantly increased the HBsAb titer after two immunizations compared to non-adjuvant or CpG ODN (Fig. 1A). Next, we assessed the ability of cGAMP to induce the cellular immune response to HBsAg. A previous study reported a peptide sequence to evaluate the MHC-I restricted cellular immune response to HBsAg (Ando et al., 1993). We therefore used this peptide sequence to stimulate splenocytes from immunized mice in the ELISPOT assay. As shown in Fig. 1B, the mice immunized with cGAMP mounted strong cellular responses against HBsAg, as indicated by the significant expansion of IFN-γ-producing cells in response to *ex vivo* re-stimulation with HBsAg peptides. Moreover, we measured the HBsAb titer and the frequency of IFN-γ-producing cells in female mice after the immunizations with r-HBsAg and cGAMP. The administration with r-

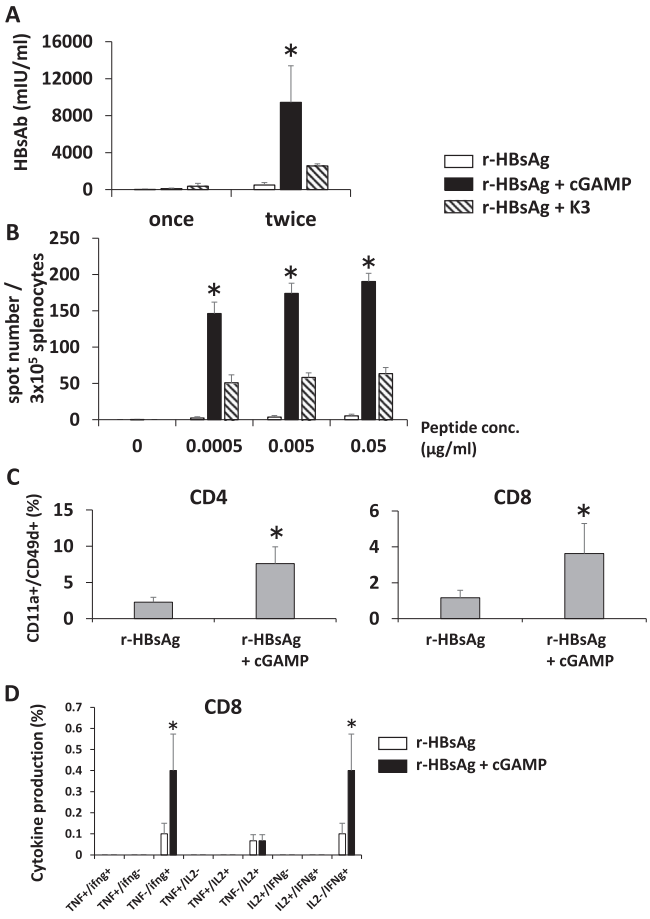

Fig. 1. cGAMP enhances the humoral and cellular immune response to HBsAg vaccination in WT mice. WT mice were immunized twice at a one interval with r-HBsAg (3 μg/mouse) (n = 4), r-HBsAg (3 μg/mouse) + cGAMP (2 μg/mouse) (n = 4), or r-HBsAg (3 μg/mouse) + CpG ODN (10 μg/mouse) (n = 4). Serum was obtained from the immunized mice 7 and 14 days after the immunization. Splenocytes were isolated from the immunized mice 7 days after the second immunization. (A) The concentration of HBsAb in serum from the mice immunized once and twice. The results are shown as the mean ± SEM (4 mice/group). (B) Splenocytes from the mice immunized twice were stimulated *ex vivo* with the peptides IPQSLDSWWTSL (0–0.05 μg/ml) and monitored for IFN-γ-secreting cells by means of an ELISPOT assay. The results are shown as the mean ± SEM (4 mice/group). (C) The percentage of $CD4^+$/$CD11a^{high}$/$CD49d^{high}$ and $CD8^+$/$CD11a^{high}$/$CD49d^{high}$ cells in the spleen after two immunizations with r-HBsAg alone or r-HBsAg + cGAMP. The results are shown as the mean ± SEM (4 mice/group). (D) Flow cytometry staining for intracellular cytokines TNF-α, IFN-γ, and IL-2. The cells were gated for CD8-positive cells. *indicates statistically significant differences; $P < 0.05$.

HBsAg and cGAMP also enhanced the cellular and humoral immune response in female mice (Supplementary Fig. 1). Antigen-specific T cells are characterized by CD11a and CD49d double positivity (Christiaansen et al., 2017; Hwang et al., 2016). Therefore, we stained the splenocytes from the mice immunized twice with anti-CD11a and anti-CD49d antibody. The frequency of CD11a and CD49d double positive cells among CD4 and CD8 T cells significantly increased in the mice immunized with r-HBsAg and cGAMP (Fig. 1C). The intra cellular staining also indicated that CD8 T cells from the mice administered r-HBsAg and cGAMP markedly increased the production of IFN-γ compared to mice immunized with r-HBsAg alone (Fig. 1D). Moreover, we examined the ability of cGAMP to induce the cellular immune response to HBsAg in IHLs. As shown in Supplementary Fig. 2, cGAMP induced the significant expansion of IFN-γ-producing cells in response to *ex vivo* re-stimulation with HBsAg peptides on IHLs. The frequency of CD11a and CD49d double positive cells among CD4 and CD8 T cells in IHLs also increased in the mice immunized with r-HBsAg and cGAMP.

3.2. Cytokine and chemokine production by the spleen in response to the injection of HBsAg and cGAMP

Several cytokines play critical roles in the activation and proliferation of antigen-specific CTLs (Bachmann et al., 1995; Ito et al., 2008; Widmer and Grabstein, 1987). Accordingly, we measured the mRNA levels of IFN-β, IFN-γ, IL-2, IL-4, IL-12b, IL-17a, and TNF-α in spleen from mice 24 h and 48 h after the immunization (Fig. 2). The expression of IFN-β, IL-2, IL-4, and TNF-α in the spleen of mice immunized with r-HBsAg and cGAMP was enhanced compared to mice immunized with r-HBsAg alone. Moreover, we measured the mRNA expression of several chemokines related to the induction of an antigen specific immune response in the spleen after the immunization. mRNA expression of CCL2 and CXCL10 was significantly enhanced by the inoculation with r-HBsAg + cGAMP (Fig. 2). We also measured the mRNA levels of these cytokines and chemokines in the liver from mice 24 h and 48 h after the immunization (Supplementary Fig. 4). The immunization with cGAMP did not affect the expression of these cytokines and chemokines significantly.

3.3. Phenotype of lymphocytes in spleen and DLN after immunization

In general, the induction of an antigen-specific cellular immune response after vaccination is involved in the activation of antigen presenting cells (APCs) such as dendritic cells (DCs). Therefore, we evaluated the frequency and activation of APCs in the spleen and draining lymph nodes (DLNs) after the immunization (Fig. 3A-D). The immunization with r-HBsAg + cGAMP increased the population of $CD11c^+$ cells in spleen compared to r-HBsAg alone (Fig. 3A). On the other hand, the expression of CD40, CD86, and MHC-II which are the activation marker of DCs in spleen was not enhanced by the administration with cGAMP (data not shown). The frequency of $CD11c^+$ and $CD11b^+$ cells in DLNs also increased after the inoculation with cGAMP (Fig. 3B). Moreover, the expression of CD40 and CD86 in these cells was enhanced by the administration with cGAMP in DLNs (Fig. 3C and D). Next, we measured the proportion of CD4 or CD8 positive cells in the spleen 1 and 3 days after the inoculation with r-HBsAg alone or r-HBsAg + cGAMP. There was no difference between these groups in the number of splenocytes or the rate of CD4- or CD8-positive cells (data not shown). However, the expression of CD69 on CD4 and CD8 T cells 1 day after inoculation was up-regulated by the administration with cGAMP (Fig. 3E).

3.4. cGAMP enhances HBsAg-specific immune response in HBV-Tg mice

HBV-Tg mice are similar to the patients with chronic HBV infection in the immune tolerance to HBV-specific antigen. Therefore, we also evaluated the ability of cGAMP to induce HBsAg-specific cellular and humoral immune responses in HBV-Tg mice. HBV-Tg mice were immunized with r-HBsAg or r-HBsAg + cGAMP on day 0 and day9. HBsAb and HBsA in HBV-Tg mice were measured after immunization. As shown in Fig. 4A, HBsAb levels in HBV-Tg mice administered r-HBsAg + cGAMP significantly increased compared to the mice treated with r-HBsAg alone 15 days after first immunization. HBsAg concentration significantly decreased after the immunization with r-HBsAg + cGAMP (Fig. 4B). By contrast, the immunization with HBsAg alone did not affect the concentration of HBaAg in HBV-Tg mice. To evaluate the HBsAg-specific cellular immune response, we performed an ELISPOT assay in HBV-Tg mice immunized twice. Splenocytes from the immunized HBV-Tg mice were stimulated with HBs peptides at a high concentration compared to those from WT mice, because HBV-Tg mice have tolerance to HBsAg. As shown in Fig. 4C, the HBV-Tg mice immunized with cGAMP mounted strong cellular responses against

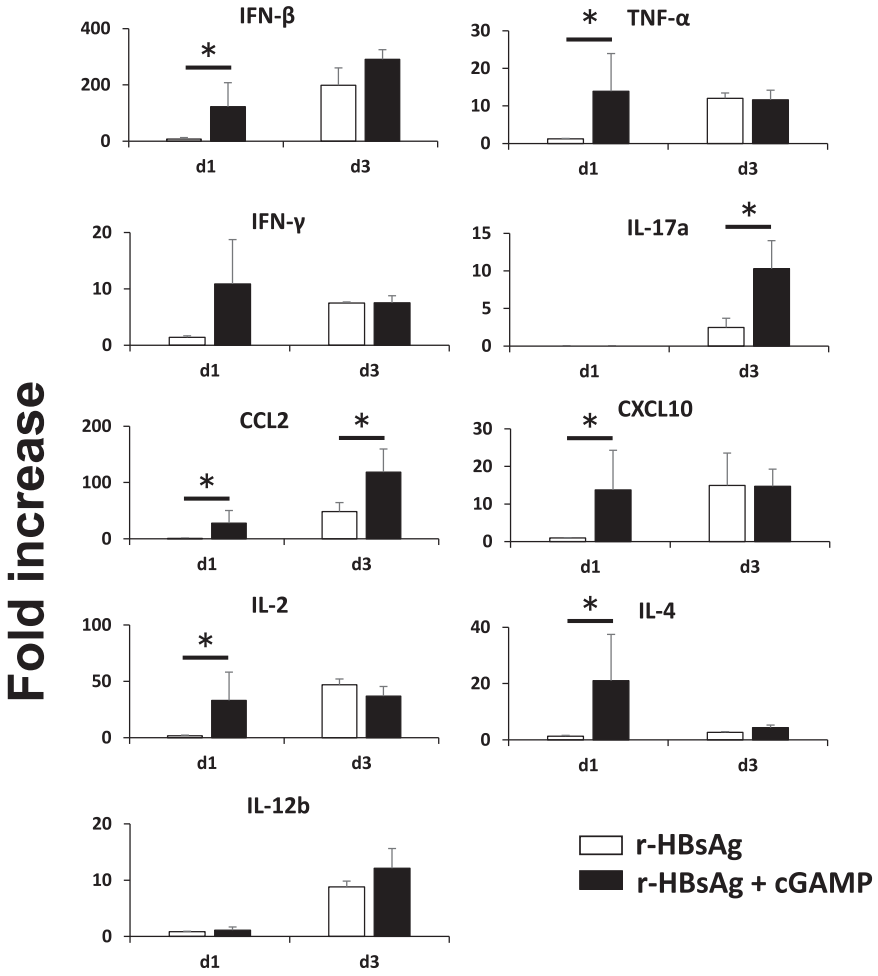

Fig. 2. Cytokine and chemokine expression in spleen in response to the injection of HBsAg and cGAMP. The relative expression levels of IFN-β, IFN-γ, TNF-α, IL-2, IL-4, IL-12b, IL-17a, CCL2, and CXCL10 mRNA in the spleen of the mice were measured using quantitative real-time RT-PCR (4 mice/group). The results were normalized to 18S rRNA. Each data point and error bar represent the mean and SEM, respectively, of data from triplicate samples. *indicates statistically significant differences; $P < 0.05$.

HBsAg, as indicated by the significant expansion of IFN-γ-producing cells in response to *ex vivo* re-stimulation with HBsAg peptide. Next, we evaluated the histological examination of the liver tissues after the immunization. In Fig. 4D, focal inflammatory foci were observed in the liver of HBV-Tg mice immunized with r-HBsAg + cGAMP. Inflammatory cell foci are defined as the existence of more than ten inflammatory cells. Moreover, the number of focal inflammatory foci in the mice immunized with r-HBsAg + cGAMP significantly increased compared to mice treated with r-HBsAg alone.

4. Discussion

In the present study, we examined the adjuvant effect of cGAMP in HBV vaccination. The administration with cGAMP up-regulated several cytokines that are critical for the induction of the antigen-specific immune response in the spleen. Moreover, the activation of APCs such as DCs were enhanced after cGAMP injection. As a result, cGAMP markedly enhanced the cellular and humoral immune responses to HBV vaccination. Although HBV-Tg mice are tolerant to HBV-antigen, the vaccination with r-HBsAg and cGAMP induced HBV-specific cellular and humoral immunity even in HBV-Tg mice.

cGAMP binds and activates the adaptor protein STING. STING in turn activates the protein kinases IKK and TBK1, leading to activation of the transcription factors NF-kB and IRF3, respectively. The activation of NF-kB and IRF3 induces the enhancement of immune and inflammatory gene products including type I IFN and TNF-α. Therefore, the STING pathway is one of the critical immune stimulators in various diseases such as, cancer and infectious diseases. Recently, the anti-tumor effect of cGAMP was evaluated in several studies. Intratumoral administration with cGAMP has promoted tumor regression in several tumor-bearing mice models (Li et al., 2016; Ohkuri et al., 2017). The Th1 immune response is critical for its anti-tumor effect. cGAMP as adjuvant powerfully induces the Th1 immune response in tumor-bearing mice and influenza mice (Junkins et al., 2018). In the present study, HBsAg-specific IFN-γ production in CD8 T cells was also enhanced by the administration of r-HBsAg and cGAMP (Fig. 1B). Vaccination with OVA and cGAMP markedly enhances OVA-specific IgG1 in the serum of immunized mice (Li et al., 2013). Here, the combined immunization with r-HBsAg and cGAMP also significantly increased the concentration of HBsAb in the serum (Fig. 1A). These results indicate that cGAMP strongly increases both Th1 and Th2 responses in HBV vaccination.

The induction of an antigen-specific immune response is affected by DCs and Th1/Th2-related cytokines and chemokines. cGAMP promotes the activation of DCs and the accumulation of DCs in tumor sites (Li et al., 2016). Here, the combined vaccination with r-HBsAg and cGAMP increased the number of CD11b and CD11c positive cells in the spleen and DLNs (Fig. 3A and B). In DLNs, the activation markers of APCs such as CD40 and CD86 increased after cGAMP administration (Fig. 3C and D). The activation of APCs in lymphoid tissues was involved in the enhancement of immune response by the administration with cGAMP.

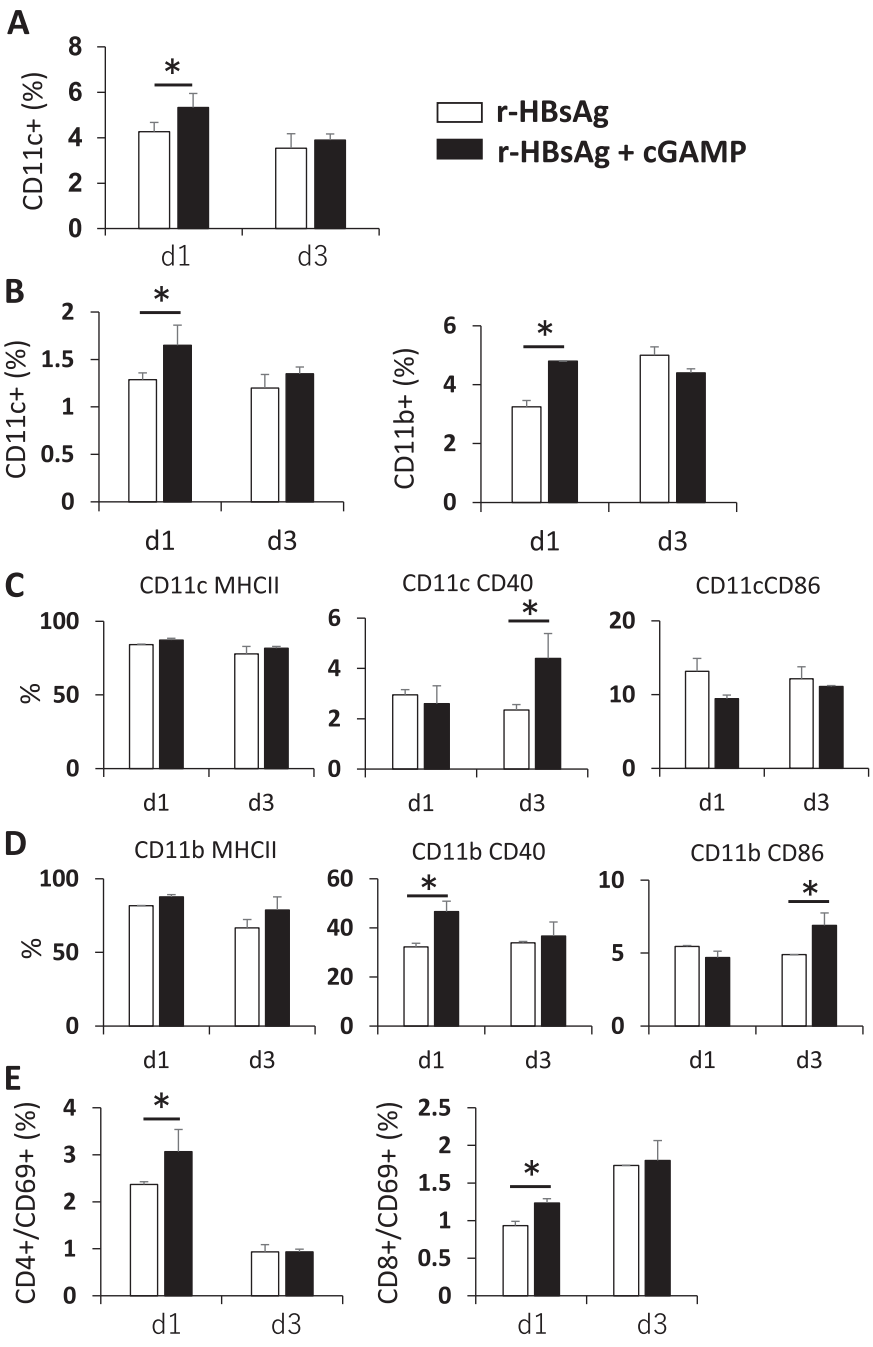

Fig. 3. Phenotype of lymphocytes in spleen and draining lymph nodes (DLNs) after immunization with cGAMP. WT mice were immunized once with r-HBsAg or r-HBsAg + cGAMP (3–4 mice/group). Splenocytes or lymphocytes in DLNs were isolated from the immunized mice 1 or 3 days after the immunization. (A) The percentage of $CD11c^+$ cells in the spleen after immunization. (B) The percentage of $CD11c^+$ or $CD11b^+$ cells in DLNs after immunization. (C) The expression of CD40, CD86 and MHC class II on $CD11c^+$ cells in DLNs was analyzed after immunization. (D) The expression of CD40, CD86 and MHC class II on $CD11b^+$ cells in DLNs was analyzed after immunization. (E) The percentage of $CD69^+$ cells among $CD4^+$ or $CD8^+$ cells of the spleen after the immunization. *indicates statistically significant differences; $P < 0.05$.

The experiment in vitro also revealed that DCs was required to increase the frequency of antigen-specific T cells after the stimulation with HBsAg and cGAMP (Supplementary Fig. 3). Previous studies indicated that the activation of DCs after cGAMP treatment was involved in the production of antigen-specific antibody via the activation of Tfh and Th2 cells (Na et al., 2016; Shin et al., 2015). Therefore, the enhancement of HBsAg-specific antibody may be dependent on the activation of APCs after the immunization with cGAMP partially. Moreover, cGAMP activated CD4 and CD8 T cells early after vaccination, and the activation of these cells may have contributed to the induction of the Th1 and Th2 immune response (Fig. 3E). The expression of IL-2, IL-4, TNF-α, CCL2, and CXCL10 significantly increased after the vaccination with r-HBsAg and cGAMP (Fig. 2). In general, IL-2 is critical for the induction of antigen-specific immune responses. The deficiency of TNF-α impairs the induction of HBsAg-specific CTL in DNA immunization (Kasahara et al., 2003). Previous study demonstrated that antigen-specific antibody production was impaired in TNFR knockout mice (Le Hir et al., 1996). IL-4 is also involved in the induction of the Th2 immune response. The increase of TNF-α and IL-4 production may be involved in the enhancement of HBs-specific antibody levels. CCL2 and CXCL10 are critical chemokines to induce the antigen-specific immune response (Bacher et al., 1996; Berencsi et al., 2011; Thapa et al., 2008). Thus, several cytokines and chemokines contribute to the enhancement of Th1/Th2 immune responses after administration of cGAMP.

HBV-Tg mice are a suitable animal model for the HBV carrier state because HBV-Tg mice harbor HBV from the neonatal period, and express HBV-related antigen in sera and liver throughout their lives (Akbar and Onji, 1998). HBsAg-specific lymphocytes are not detected in these HBV-Tg mice in situ. Moreover, vaccination with HBsAg and complete Freund's adjuvant dose not induce the production of anti-HBsAb and HBsAg-specific lymphocytes in HBV-Tg mice (Akbar et al., 1993). In our previous studies, the vaccination with r-HBsAg + alpha-

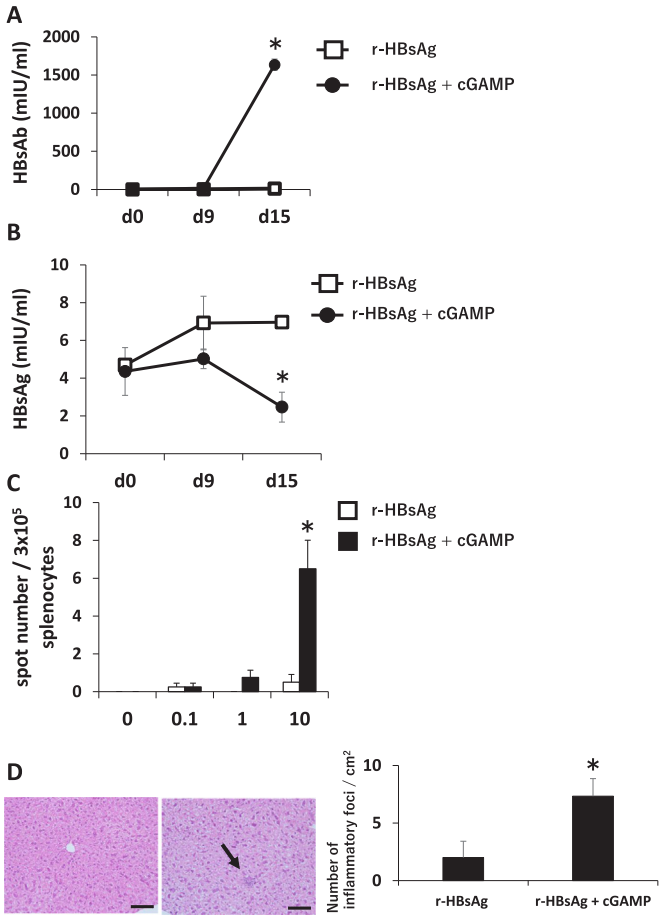

Fig. 4. cGAMP enhances the humoral and cellular immune response to HBsAg vaccination in HBV-Tg mice. HBV-Tg mice were twice immunized with r-HBsAg or r-HBsAg + cGAMP (4 mice/group). Serum was obtained from the mice 0, 9, and 15 days after the first immunization. (A) HBsAb and (B) HBsAg in the serum were measured after immunization. (C) HBV-Tg mice were immunized twice (days 0 and 9). Splenocytes were isolated from the immunized mice 6 days after the second immunization. Splenocytes were stimulated *ex vivo* with the HBsAg S28–39 peptide (0–10 μg/ml) and monitored for IFN-γ-secreting cells by means of an ELISPOT assay. The results are shown as the mean ± SEM (4 mice/group). (D) Representative photomicrographs of liver sections 6 days after second immunization stained with hematoxylin-eosin (left panel). Scale bars: 100 μm. Black arrow indicates the focal inflammatory foci. The number of inflammatory foci is shown in right panel. *indicates statistically significant differences; $P < 0.05$.

galactosylceramide or r-HBsAg + IDO inhibitor also did not induce an immune response against HBsAg in HBV-Tg mice *ex vivo*. In the present study, two immunizations with r-HBsAg and cGAMP significantly increased the production of anti HBsAb in the serum (Fig. 1A). Moreover, the concentration of HBsAg significantly decreased in HBV-Tg mice after the vaccination with r-HBsAg and cGAMP (Fig. 1B). These data indicate that vaccination with cGAMP can induce functional anti-HBsAb even in HBV-Tg mice. In addition, HBsAg-specific IFN-γ production in CD8 T cells in HBV-Tg mice was enhanced after the administration of cGAMP. Moreover, histological examination revealed that the number of focal inflammatory foci in HBV-Tg mice immunized with r-HBsAg + cGAMP increased compared that mice immunized with r-HBsAg alone. The increase in these foci may reflect the enhancement of the HBV-specific cellular immune response in HBV-Tg mice. Thus, the immunization with r-HBsAg and cGAMP can induce anti-HBsAb and HBsAg-specific cellular immunity even in HBV-Tg mice that have immunogenic tolerance to HBsAg.

Recently, the US FDA approved Heplisav (rHBsAg-1018 ISS) for HBV vaccination (Hyer et al., 2018). Heplisav utilizes a novel Toll-like receptor 9 (TLR9) agonist as adjuvant mixed with r-HBsAg. In three pivotal, phase 3 trials, Heplisav induced significantly higher and earlier seroprotection rates in adult populations (Halperin et al., 2012; Heyward et al., 2013; Jackson et al., 2018). In the present study, the vaccination including CpG ODN also increased the concentration of HBsAb in serum compared to the vaccination without CpG ODN. Moreover, cGAMP increased HBsAb concentration in the serum of WT mice after the immunization compared to CpG ODN. cGAMP also enhanced HBsAg-specific cellular immune response compared to CpG ODN (Fig. 1A). Thus, the vaccination with cGAMP markedly up-regulated cellular and humoral immune response compared to CpG ODN, and cGAMP will be expected to be part of new vaccination strategies. Moreover, the vaccination with cGAMP increased HBsAb in the serum of HBV-Tg and HBsAg-specific IFN-γ production in HBV-Tg which are tolerant to HBV-related antigen. The therapeutic goal for chronic HBV infection is seroclearance of HBsAg (EASL, 2017; Lok and McMahon, 2007). The reduction of serum HBsAg remains the emerging therapeutic target in several studies (Lampertico et al., 2015; Terrault et al., 2016). In the present study, cGAMP reduced HBsAg in the serum of HBV-Tg mice. These results indicate that therapeutic vaccination may eliminate HBV from the patient chronically infected with HBV.

In conclusion, we evaluated the effect of cGAMP in HBV vaccination and demonstrated that the vaccination with cGAMP induced an HBV-specific cellular and humoral immune response in WT mice. These effects were observed in HBV-Tg mice. Vaccination with cGAMP may lead to strategies to eliminate HBV completely from patients.

Acknowledgments

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Conflict of interest

The authors have no financial or commercial conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.03.013.

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