IL-12–Based Vaccination Therapy Reverses Liver-Induced Systemic Tolerance in a Mouse Model of Hepatitis B Virus Carrier

Zhutian Zeng, Xiaohui Kong, Fenglei Li, Haiming Wei, Rui Sun and Zhigang Tian

*J Immunol* 2013; 191:4184-4193; Prepublished online 18 September 2013; doi: 10.4049/jimmunol.1203449

http://www.jimmunol.org/content/191/8/4184

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/09/18/jimmunol.1203449.DC1

Why *The JI*?

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

References

This article cites 40 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/191/8/4184.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852 Copyright © 2013 by The American Association of Immunologists, Inc. All rights reserved. Print ISSN: 0022-1767 Online ISSN: 1550-6606.
GL-12–Based Vaccination Therapy Reverses Liver-Induced Systemic Tolerance in a Mouse Model of Hepatitis B Virus Carrier

Zhuitian Zeng,* Xiaohui Kong,* Fenglei Li,* Haiming Wei,*† Rui Sun,*† and Zhigang Tian*†

Liver-induced systemic immune tolerance that occurs during chronic hepatadnavirus infection is the biggest obstacle for effective viral clearance. Immunotherapeutic reversal of this tolerance is a promising strategy in the clinic but remains to be explored. In this study, using a hepatitis B virus (HBV)-carrier mouse model, we report that IL-12–based vaccination therapy can efficiently reverse systemic tolerance toward HBV. HBV-carrier mice lost responsiveness to hepatitis B surface Ag (HBsAg) vaccination, and IL-12 alone could not reverse this liver-induced immune tolerance. However, after IL-12–based vaccination therapy, the majority of treated mice became HBsAg+ in serum; hepatitis B core Ag was also undetectable in hepatocytes. HBV clearance was dependent on HBsAg vaccine-induced anti-HBV immunity. Further results showed that IL-12–based vaccination therapy strongly enhanced hepatic HBV-specific CD8+ T cell responses, including proliferation and IFN-γ secretion. Systemic HBV-specific CD4+ T cell responses were also restored in HBV-carrier mice, leading to the arousal of HBsAg-specific follicular Th-germinal center B cell responses and anti-hepatitis B surface Ag Ab production. Recovery of HBsAg-specific responses also correlated with both reduced CD4+Foxp3+ regulatory T cell frequency and an enhanced capacity of effector T cells to overcome inhibition by regulatory T cells. In conclusion, IL-12–based vaccination therapy may reverse liver-induced immune tolerance toward HBV by restoring systemic HBV-specific CD4+ T cell responses, eliciting robust hepatic HBV-specific CD8+ T cell responses, and facilitating the generation of HBsAg-specific humoral immunity; thus, this therapy may become a viable approach to treating patients with chronic hepatitis B. The Journal of Immunology, 2013, 191: 4184–4193.

The liver favors induction of immune tolerance rather than immunity during chronic infection with hepatotropic pathogens (1), such as hepatitis B virus (HBV). HBV infection is one of the most serious health concerns worldwide, because chronically infected individuals are predisposed to development of severe liver cirrhosis and hepatocellular carcinoma (2, 3). Lifelong viral persistence in chronic hepatitis B (CHB) carriers is due to liver-induced immune tolerance toward HBV, which is characterized by defective HBV-specific T cell responses and undetectable anti–hepatitis B surface Ag Ab (anti-HBs) levels (4). Reversing this immune tolerance will therefore be a critical step toward completely eliminating HBV from the host (5, 6).

Therapeutic vaccination against HBV is a promising approach to reverse HBV tolerance in chronically infected individuals (6, 7). Previous animal studies revealed that immunization with a hepatitis B surface Ag (HBsAg) vaccine resulted in decreased HBV titers and the generation of anti-HBs in HBV-transgenic (tg) mice (8, 9). However, clinical studies using these strategies revealed poor therapeutic effects in CHB patients in an immunotolerant phase (10, 11). The divergence in therapeutic outcome may be due, in part, to differences in the type of tolerance induced between HBV-tg mice and CHB patients (12, 13). Therefore, a mouse model that more accurately mimics HBV-induced “acquired” tolerance during CHB infection could aid in developing immunotherapeutic strategies against HBV. Recently, an immunocompetent HBV-carrier mouse model was established using hepatic gene transfer of an HBV-containing plasmid by hydrodynamic injection. Because these mice are completely unresponsive to peripheral HBsAg vaccination because of liver-induced systemic tolerance, they provide a better mouse model with which to study liver tolerance during HBV persistence (12).

The transient and insufficient effects of vaccine therapy alone in clinical studies also suggest the need for combination therapy (5, 6). On one hand, combining HBsAg vaccine with an immunostimulant, such as CpG, exhibited improved efficacy to break HBV tolerance via Th1-biased mechanisms (14). On the other hand, combining HBsAg vaccine with an antiviral agent, including lamivudine, was favored in other trials, because HBV-specific T cell hyporesponsiveness during CHB infection always correlated with high viral load (15). However, neither approach was completely effective in the clinic, suggesting the possibility that an agent possessing both immunostimulatory and antiviral effects could optimize vaccination therapy in combination with a vaccine.
IL-12 is a typical proinflammatory cytokine that plays a critical role in host defense against pathogens, including HBV (16). Impaired IL-12 secretion by dendritic cells is implicated in contributing to the chronicity of HBV infection (17). Importantly, IL-12 can also inhibit HBV replication in HBV-tg mice by inducing IFN-γ production (18). However, the antiviral effect of IL-12 alone does not appear to be more effective than other traditional drugs in clinical trials (19). In this study, we used an HBV-carrier mouse model to study the effect of IL-12 on overcoming liver-induced systemic tolerance. Although neither IL-12 nor HBV vaccine alone could efficiently eliminate HBV, a combination treatment using both HBsAg vaccine and IL-12 led to effective HBV clearance.

Materials and Methods

Mice

Five-week-old male C57BL/6 mice were purchased from SLAC Laboratory Animal Corporation (Shanghai, China). HBV-tg mice were purchased from the Infectious Disease Center of the No. 458 Hospital (Guangzhou, China). Rag1−/− mice were purchased from the Model Animal Research Center (Nanjing, China). CD4−/− mice were a kind gift from Dr. Zhexiong Lian (University of Science and Technology of China). All mice were maintained under specific pathogen-free conditions and used in accordance with the Guide for the Care and Use of Laboratory Animals.

HBV-carrier mouse model

The pAAV-HBV1.2 plasmid, containing the full-length HBV DNA, was kindly provided by Dr. Ding-Shinn Chen (National Taiwan University College of Medicine, Taipei, Taiwan). To establish the HBV-carrier mouse model, we injected C57BL/6 mice (5-wk-old male mice) with 5 μg pAAV-HBV1.2 plasmid using a hydrodynamic tail-vein injection approach. Six weeks later, mice with serum HBsAg levels of at least 200 ng/ml were considered to be HBV-carrier mice.

IL-12 treatment and immunization

HBV-carrier mice were screened out 6 wk after HBV plasmid injection and s.c. injected with 100 ng IL-12 (in 200 μl total volume of PBS; Peprotech, Rocky Hill, NJ) daily for 3 consecutive days, followed by s.c. immunization with 2 μg recombinant HBsAg vaccine (adw subtype; Biokangtai Company, Shenzhen, China) in the presence of 100 ng IL-12 as a coadjuvant. This treatment regimen was repeated weekly for 3 wk. In some experiments, HBsAg vaccine was replaced by recombinant HBsAg protein (HyTest, Turku, Finland) emulsified in aluminum hydroxide (alum) (Imject Alum, Pierce, IL). Blood samples were obtained weekly by tail bleeding, and sera were kept at −20°C until use.

**FIGURE 1.** IL-12–based vaccination therapy efficiently eliminated HBV in HBV-carrier mice. (A) HBV-carrier mice were screened out 6 wk after HBV plasmid injection and were treated with IL-12–based vaccination therapy (as shown), HBsAg vaccination alone, or no treatment. (B) HBsAg levels were monitored in PBS+HBsAg vaccine–treated, IL-12+HBsAg vaccine–treated, and untreated control mice. Data represent the pooled results from three separate experiments (each point represents one mouse). (C) The ratio of HBsAg-seropositive mice was analyzed. Mice exhibiting HBsAg levels <2 ng/ml were considered HBsAg−. (D) Serum HBcAg levels and (E) HBV DNA copies were measured in each group 3 wk after treatment initiation. The cutoff value was 1 national clinical unit per milliliter (NCU/ml). (F) Immunohistochemical staining of HBcAg in liver sections from HBV-carrier mice treated with HBsAg vaccination alone or IL-12–based vaccination therapy. Micrographs (original magnification ×200) are representative liver sections from each group at week 4 after treatment initiation. A liver section from an HBV-carrier mouse that was screened out 6 wk after HBV injection and before treatment initiation was used as the control. (G) Serum ALT levels were monitored during treatment. ALT levels <28 IU/l were considered physiologically normal. Data are representative results from three independent experiments for (D), (E), and (G), with each group comprising 7–10 mice, and are shown as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. N.S., No significant difference.
Radioimmunoassay
Serum HBsAg, hepatitis B e Ag (HBeAg), anti-HBs, and anti-HBe levels were measured using the corresponding radioimmunoassay kits (North Institute of Biological Technology, Beijing, China) according to the manufacturer’s instructions.

HBV DNA detection
HBV DNA copies were detected by probe-based quantitative PCR using a diagnostic kit for HBV DNA (KHB, Shanghai, China) according to the manufacturer’s instructions.

Immunohistochemistry
Paraffin-embedded, 6-μm-thick liver sections were stained with rat anti-hepatitis B core protein (anti-HBcAg; Dako, Glostrup, Denmark) and visualized using a diaminobenzidine peroxidase substrate kit (Vector, Burlingame, CA).

ELISPOT assay
HBsAg-specific IgG ELISPOT detection was performed using a mouse IgG ELISPOT kit (MABTech, Stockholm, Sweden). In brief, prewetted polyvinylidene difluoride plates were coated with 5 μg HBsAg in sterile PBS overnight at 4°C. The plates were blocked by adding 200 μl/well RPMI 1640 medium containing 10% FCS for at least 1 h. Then, 5 × 10⁶ splenocytes in suspension (prepared under sterile conditions) were added to the wells and incubated for an additional 24 h at 37°C without any movement of the plate. After incubation, detection was performed according to the manufacturer’s instructions. HBsAg- or HBeAg-specific IFN-γ–secreting CD4⁺ T cells were detected using a mouse IFN-γ ELISPOT kit (Dakewe, Shenzhen, China). In brief, 1 × 10⁶ irradiated (20 Gy) syngeneic splenocytes were seeded into 96-well plates precoated with anti–IFN-γ in the presence of 5 μg/ml HBsAg or 2 μg/ml HBeAg (ID LABS, London, Canada) for 1 h at 37°C. Then, 1 × 10⁶ purified splenic CD4⁺ T cells were added to the wells and incubated for 24 h. ELISPOT detection was then performed according to the manufacturer’s instructions. All ELISPOT data were obtained and imaged by the plate-reading service provided by the Dakewe Company.

FIGURE 2. IL-12–based vaccination therapy reversed HBV tolerance by inducing anti-HBV adaptive immunity. (A) Serum HBsAg levels and (B) the ratios of HBsAg-seropositive mice were analyzed in HBV-carrier mice after IL-12–based vaccination therapy. IL-12 monotherapy, HBV vaccine therapy, or no treatment. HBsAg-emulsified with alum replaced HBsAg vaccine, and alum was also added to the IL-12 monotherapy group. (C) HBV-carrier mice that received IL-12–based vaccination therapy. IL-12 monotherapy, or HBsAg vaccine were reinjected with pAAV/HBV1.2 plasmid (5 μg) 6 wk after treatment initiation. Serum HBsAg levels were detected 1 wk after injection. (D) A total of 1 × 10⁶ splenocytes from HBV-carrier mice that received either IL-12–based vaccination therapy or HBsAg vaccine were adoptively transferred into HBV-carrier Rag1⁻/⁻ mice 6 wk after treatment initiation. Serum HBsAg levels in recipient mice were measured after 2 wk. (A) Data are shown as the mean ± SEM (unpaired t test). (C and D) Each point represents one mouse. All experiments were repeated twice with similar results. N.S., No significant difference.

Cell isolation
Hepatic mononuclear cells and splenocytes were separated as previously described (20). In brief, the murine liver was removed, cut into small pieces, and passed through a 200-mesh gauge. After a 5-min incubation at 4°C, the cells were pelleted, resuspended in 3 ml of a 40% Percoll solution, and added onto 2 ml of a 70% Percoll solution. The cells were then centrifuged at 1260 × g for 30 min. The hepatic mononuclear cells at the interface between the two Percoll gradients were collected and washed twice at 400 × g for 5 min. The spleen was removed and passed through a 200-mesh gauge. Splenocytes were then harvested after RBC lysis and washing. For CD4⁺CD25⁺ and CD4⁺CD25⁻ cell isolation, splenocytes were sorted by MACS separation using a CD4⁺CD25⁺ regulatory T cell (Treg) isolation kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer’s instructions. The purities of the isolated CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were >90 and 85%, respectively. In a parallel experiment, both CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were sorted using FACS to achieve 95% purity. Total CD4⁺ T cell isolation was performed by MACS separation using a mouse CD4⁺ T cell isolation kit (Miltenyi Biotech). For isolating CD11c⁺CD8α⁺ and CD11c⁻CD8α⁻ cells, splenocytes were labeled with PerCP-Cy5.5–anti-CD8α, PE-Cy7–anti-CD11a, and allophycocyanin–anti-CD3, and then the labeled cells were sorted by FACS.

Flow cytometry
Cells in a single-cell suspension were incubated with anti-CD16/32 (2.4G2; BD Biosciences, San Jose, CA) to block any nonspecific staining before staining with the specific Abs. Foxp3 and Ki67 were stained using a Foxp3 staining kit (eBioscience, San Diego, CA). In brief, cells were fixed, permeabilized, and then stained with Alexa 647–anti-Foxp3 or Alexa 647–anti-Ki67 (eBioscience). For intracellular IFN-γ and TNF-α staining, cells were stimulated with 30 ng/ml PMA (Sigma, St. Louis, MO) and 1 μg/ml ionomycin (Sigma) for 4 h. Monensin (2 μg/ml; Sigma) was added at the beginning of stimulation to prevent cytokine secretion. For staining the Ag-specific CD8⁺ T cells, HBsAg (ILSPIFLPLL) and HBcAg (MGLKFRQL) peptide-specific H-2Kb pentamers (Proimmune, Oxford, U.K.) were used according to the manufacturer’s instructions. The following fluorescently conjugated mAbs were used in this study: FITC–anti-GL7, FITC–anti-PDL1, FITC–anti-CD25, PE–anti-TNF-α, PE–anti-IFN-γ, PE–anti-Fas, PE–anti-Tim3, PE–anti-2B4, PE–anti-Lag3, PE–anti-CD49d, PerCP-Cy5.5–anti-CD8, PE–anti-CD69, and PE–anti-CD25.
PerCP-Cy5.5–anti-B220, PE-Cy7–anti-NK1.1, PE-Cy7–anti-CD11a, allophycocyanin–anti-CD3, allophycocyanin-streptavidin, allophycocyanin–Cy7–anti-CD4, Biotin-CXCR5 (all from BD Biosciences), and PE-Helios (Biolegend). All data were collected using a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6 software.

[^3H]thymidine incorporation and Treg induction

For evaluating HBsAg-specific proliferation, mouse splenocytes were separated and cultured (2 × 10^5 cells/well) in a 96-well U-bottom plate with 200 μl RPMI 1640 (supplemented with 10% FCS, 5 × 10^-2 mM 2-ME, 2 mM l-glutamine, and 1% penicillin/streptomycin) for 72 h in the presence of 5 μg/ml HBsAg. An unstimulated control was incubated under the same conditions in the absence of Ag.[^3H]thymidine (TdR) (1 μCi/well; Amersham Pharmacia Biotech, Amersham, U.K.) was added during the last 16 h for optimal incorporation. Data were collected using β-liquid scintillation analyzer (Perkin Elmer, Norwalk, CT) and expressed as a stimulation index (SI = cpm of HBsAg-stimulated cells/cpm of the unstimulated control). To evaluate the suppressive effect of Tregs, we cocultured 1 × 10^5 CD4+CD25- cells with CD4+CD25+ cells at a ratio of 4:1 or 2:1 in the presence of anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) for 72 h.[^3H]TdR incorporation was determined as described earlier. For Treg induction in vitro, a total of 2 × 10^5 CD4+CD25- splenocytes was cultured in the presence of anti-CD3 (2 μg/ml), anti-CD28 (1 μg/ml), TGF-β (5 ng/ml; Peprotech), and IL-2 (100 U/ml; Huaxin, Shanghai, China) for 96 h.

Statistical analysis

Statistical differences were first determined using a one-way ANOVA test to compare more than two groups. Nonpaired Student t tests were then performed to analyze the differences between two groups. For analyzing the ratio of HBsAg-seropositive mice, the log rank test was used to determine statistical differences. Differences achieving a p value <0.05 were considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

IL-12–based vaccination therapy efficiently eliminated HBV in HBV-tolerant mice

Previous studies showed that mice hydrodynamically injected with HBV plasmid represented a model of chronic HBV infection (12). Approximately 30% of these mice showed very slow spontaneous viral clearance and exhibited both persistent HBV replication in the liver and HBV surface antigenemia in the serum for >4 mo; these mice could be screened out 6 wk after plasmid injection based on the high levels of HBsAg in serum (Supplemental Fig. 1A–D). More importantly, these mice lost the ability to produce anti-HBs after conventional HBV vaccination (Supplemental Fig. 1E, 1F), indicating that they were systemically tolerant to HBV stimulation. Thus, we called them HBV-carrier mice to mimic the acquired tolerant state of human chronic HBV carriers. We then tested whether combined treatment with IL-12 could reverse the tolerant state toward peripheral HBV vaccination (termed IL-12–based vaccination therapy; Fig. 1A). As shown in Fig. 1B, serum HBsAg levels dramatically decreased in HBV-tolerant mice after 3 wk of IL-12–based vaccination therapy. Eight weeks after IL-12–
based vaccination therapy, almost all treated mice (>90%) became negative for the presence of serum HBsAg, whereas this complete loss of HBsAg occurred in only a few control mice (<20%; Fig. 1C). High HBsAg levels and HBV DNA titers in the serum are generally considered to be markers of active HBV replication in the liver (2, 3). In our model, we found that IL-12--based vaccination therapy led to remarkably decreased levels of serum HBsAg (Fig. 1D) and HBV DNA (Fig. 1E), indicating a dramatic inhibition of active HBV replication. Furthermore, hepatocyte-expressed HBcAg disappeared after IL-12--based vaccination therapy (Fig. 1F). In addition, the relatively low IL-12 dose used for this therapeutic approach did not induce any obvious liver injury or toxicity in any other organ (Fig. 1G and data not shown), suggesting that this therapy was nontoxic and safe. Intriguingly, IL-12--based vaccination therapy preferentially exerted a stronger ability to reduce serum HBsAg levels than combination therapy with IL-2, IL-15, or IFN- (Supplemental Fig. 2). Taken together, IL-12--based vaccination therapy successfully and safely reversed HBV tolerance in HBV-carrier mice.

**IL-12--based vaccination therapy eliminated HBV by inducing anti-HBV adaptive immunity**

We observed in our experiments that although IL-12 treatment alone (termed IL-12 monotherapy) induced a rapid decrease in serum HBsAg levels during the first 1–2 wk, it did not induce loss of serum HBsAg thereafter in HBV-tolerant mice (Supplemental Fig. 3A, 3B). Also, neither HBsAg nor IL-12 administered with alum were able to achieve similar therapeutic effects as IL-12--based vaccination therapy (with alum; Fig. 2A, 2B), indicating that HBsAg rather than the alum adjuvant played a critical role in HBV clearance from tolerant mice. In accordance with this, IL-12--based vaccination therapy, but not IL-12 monotherapy or HBsAg vaccine alone, successfully prevented HBV reconstitution in HBV-carrier mice challenged with a second HBV plasmid injection (Fig. 2C). In addition, splenocytes from IL-12--based vaccination-treated mice adoptively transferred into immunodeficient HBV-carrier Rag1−/− recipients eliminated HBV (Fig. 2D), indicating that a recall HBV-specific immune response was successfully established in HBV-tolerant mice after IL-12--based vaccination therapy.

**IL-12--based vaccination therapy reversed the tolerant state toward peripheral HBsAg vaccination**

The dominant contribution of HBsAg vaccine to HBV clearance during IL-12--based vaccination therapy prompted us to evaluate the immune responses against HBsAg vaccine in HBV-carrier mice. We observed that IL-12--based vaccination therapy reversed tolerance to peripheral HBsAg vaccination by promoting HBV-specific CXCR5- PD-1+ follicular helper T cell accumulation in the draining lymph node (dLN) (Fig. 3A), which was essential for germinal center (GC) formation (21). Consistent with this finding, GC B cells proliferated significantly more in both the dLN and spleen (Fig. 3B and data not shown) in response to IL-12--based vaccination therapy compared with HBV vaccine or IL-12 alone. Because GC B cell amplification usually causes Ag-specific Ab production and memory responses (21), we examined serum anti-HBs levels in HBV-carrier mice. As shown in Fig. 3C, HBsAg-specific, IgG-producing cells were dramatically enhanced after IL-12--based vaccination therapy, and most mice receiving IL-12--based vaccination therapy became anti-HBs+ in serum at week 8 (Fig. 3D). To confirm that HBsAg-specific T cell responses were recovered in HBV-carrier mice after IL-12--based vaccination therapy, we performed an in vitro HBsAg-specific [3H]TdR incorporation experiment. Only splenocytes from HBV-carrier mice treated with IL-12--based vaccination therapy proliferated in response to HBsAg stimulation, although this proliferation was relatively weak (Fig. 3E). Taken together, these findings clearly indicated that IL-12--based vaccination therapy could restore host responsiveness toward peripheral HBsAg vaccination in HBV-tolerant mice.
IL-12–based vaccination therapy reinvigorated the systemic HBV-specific CD4+ T cell response

In our study, IL-12–based vaccination therapy induced systemic lymphocytosis, especially in the livers of HBV-carrier mice. Investigating each specific subset individually, we found that the frequency and absolute number of hepatic adaptive immune cells, including CD4+ T, CD8+ T, and B cells, strikingly increased compared with mice treated with HBV vaccine alone or mice that did not receive treatment, which was in contrast with the observed decrease in predominant liver-resident innate immune cells, including NK and NKT cells (Fig. 4A, 4B). These results suggested that T cell responses might play a critical role in reversing HBV tolerance. Consistent with these findings, IL-12–based vaccination therapy lost the ability to eliminate HBV in HBV-carrier Rag1−/− mice deficient in both T and B cells, but it still reduced HBV to a similar level as IL-12 monotherapy, further suggesting that adaptive immunity was required to eradicate HBV, whereas HBV inhibition mediated by IL-12 alone seemed to be independent of adaptive immunity (Fig. 4C). A similar phenomenon was observed in HBV-carrier CD4−/− mice (Fig. 4D), indicating that CD4+ T cells were indispensable for complete HBV clearance during IL-12–based vaccination therapy.

To further evaluate HBV-specific T cell responses, we used a recently reported approach tracking CD11ahighCD8alo or CD49dimCD11ahigh markers to label and monitor Ag-specific CD8+ or CD4+ T cells, respectively, during viral infection (22, 23). Indeed, we observed that the kinetics of both peripheral CD11ahighCD8aloCD8+ T cells and CD49dimCD11ahighCD4+ T cells after high-dose HBV plasmid injection (mimicking acute infection) were similar to classical Ag-specific T cell kinetics during viral infection (Supplemental Fig. 4A, 4B) (24). Notably, low-dose HBV plasmid injection (mimicking HBV tolerance) did not result in such significantly upregulated integrin markers on T cells from the PBL, implying that the HBV-carrier mice lost the ability to elicit strong HBV-specific T cell responses in the periphery (data not shown).

We sorted CD8+ T cells from HBV-carrier mice, separated them into CD11ahighCD8alo and CD11ahighCD8alo subsets, and transferred them separately into a naive recipient. As expected, only the CD11ahighCD8alo donor cells robustly proliferated in response to HBV rechallenge (Supplemental Fig. 4C, 4D), indicating that the CD11ahighCD8alo cells were HBV specific. Therefore, CD11ahighCD8alo or CD49dimCD11ahigh markers could be used to monitor HBV-specific CD8+ or CD4+ T cells in our model.

CD4+ T cell responses to HBV are usually very weak in chronic HBV-infected individuals and are considered to impede induction and maintenance of HBV-specific CD8+ T cell and Ab responses (3, 4). However, in our mouse model, HBV-specific CD4+ T cells from the PBL (Fig. 5A), liver, and spleen (Fig. 5B) dramatically

**FIGURE 5.** IL-12–based vaccination therapy restored systemic HBV-specific CD4+ T cell responses in HBV-carrier mice. HBV-carrier mice were treated with IL-12–based vaccination therapy, vaccine alone, or no treatment. Three weeks later, hepatic mononuclear cells and splenocytes were harvested. (A) CD49dimCD11ahigh frequency among CD4+ T cells in PBL was monitored weekly; (B) these cells were also identified in liver (left) and spleen (right), gated on CD4+ T cells. (C) Ki67 staining of splenic CD49dimCD11ahighCD4+ T (Non-HBV) or CD49dimCD11ahighCD4+ T cells (HBV) are shown. (D) Klrg1 and (E) CD62L staining of splenic CD49dimCD11ahighCD4+ T (HBV-specific) are shown. Intracellular (F) IFN-γ and (G) TNF-α were examined using flow cytometry, gated on splenic CD49dimCD11ahighCD4+ T (HBV-specific) and CD49dimCD11ahighCD4+ T cells (HBV-nonspecific). Splenic CD4+ T cells from mice in all three groups were separated by MACS at week 3, and (H) HBcAg- and (I) HBsAg-specific IFN-γ ELISPOT assays were performed. Results are expressed as the numbers of Ag-specific IFN-γ-secreting spot-forming cells (SFC), where Ag-specific SFC numbers = SFCs in the presence of Ag stimulation − SFCs in the absence of Ag stimulation. Data are representative of three independent experiments. (A, H, and I) Data are shown as the mean ± SEM. **p < 0.01, ***p < 0.001.
increased 3 wk after IL-12–based vaccination therapy, mainly because of the selective expansion of HBV-specific CD4+ T cells (Fig. 5C). Furthermore, HBV-specific CD4+ T cells from mice treated with IL-12–based vaccination therapy expressed higher KLRG1 and lower CD62L levels (Fig. 5D, 5E), a phenotype representing effector/memory T cells (25). In line with this phenotype, splenic HBV-specific CD4+ T cell effector function, assessed by IFN-γ and TNF-α secretion, was greatly enhanced after IL-12–based vaccination therapy compared with HBV vaccine alone or untreated controls (Fig. 5F, 5G). Specifically, both HBCAg- and HBsAg-specific IFN-γ–secreting CD4+ T cells were greatly increased after IL-12–based vaccination therapy (Fig. 5H, 5I), indicating that a multispecific and robust anti-HBV CD4+ T cell response was recovered. Taken together, these data showed that IL-12–based vaccination therapy could reinvigorate systemic HBV-specific CD4+ T cell responses in HBV-tolerant mice.

**IL-12–based vaccination therapy inhibited Treg generation**

The balance between effector/memory CD4+ and regulatory CD4+ T cells may be critical for the outcome of immune responses. As expected, IL-12–based vaccination therapy reduced the frequency of CD4+Foxp3+ or CD4+CD25+ Tregs in both the liver and spleen of HBV-carrier mice (Fig. 6A, 6B). We supposed that the reduced Treg frequency was due to the inhibition of Treg induction. Indeed, the Helios− cells among the Foxp3+ Tregs, which appeared to be the induced Tregs (26, 27), were reduced after IL-12–based vaccination therapy (Fig. 6C). In addition, CD4+CD25+ T cells from HBV-carrier mice treated with IL-12–based vaccination therapy were refractory to TGF-β–induced Foxp3 expression in vitro (Fig. 6D); moreover, we also found that these CD4+CD25+ effector T cells proliferated to a greater extent than cells from vaccine-treated control mice in the presence of Tregs (Fig. 6E), indicating that IL-12–based vaccination therapy induced effector T cells to overcome Treg-mediated inhibition. Consistent with these findings, splenocytes isolated from HBV-carrier mice completely inhibited HBsAg-specific proliferation of splenocytes from HBsAg-vaccinated naive mice in vitro, whereas splenocytes from IL-12–based vaccination-treated HBV-carrier mice did not inhibit the Ag-specific proliferation of these cells (Fig. 6F). These results suggest that IL-12–based vaccination therapy diminishes the frequency of Ag-specific inhibitory cells that normally exist in HBV-carrier mice.

**IL-12–based vaccination therapy induced robust hepatic HBV-specific CD8+ T cell responses**

A robust HBV-specific CD8+ T cell response has previously been reported to be critical for HBV elimination in the clinic (3). In our study, HBV-specific CD8+ T cells actually responded more quickly and robustly than CD4+ T cells. HBV-carrier mice receiving IL-12–based vaccination therapy showed rapidly increased CD11ahi CD8+ T cell responses, mainly because of the selective expansion of HBV-specific CD8+ T cells (Fig. 5C). Furthermore, HBV-specific CD8+ T cells from mice treated with IL-12–based vaccination therapy expressed higher KLRG1 and lower CD62L levels (Fig. 5D, 5E), a phenotype representing effector/memory T cells (25). In line with this phenotype, splenic HBV-specific CD8+ T cell effector function, assessed by IFN-γ and TNF-α secretion, was greatly enhanced after IL-12–based vaccination therapy compared with HBV vaccine alone or untreated controls (Fig. 5F, 5G). Specifically, both HBCAg- and HBsAg-specific IFN-γ–secreting CD8+ T cells were greatly increased after IL-12–based vaccination therapy (Fig. 5H, 5I), indicating that a multispecific and robust anti-HBV CD8+ T cell response was recovered. Taken together, these data showed that IL-12–based vaccination therapy could reinvigorate systemic HBV-specific CD4+ T cell responses in HBV-tolerant mice.

**IL-12–based vaccination therapy inhibited Treg generation**

The balance between effector/memory CD4+ and regulatory CD4+ T cells may be critical for the outcome of immune responses. As expected, IL-12–based vaccination therapy reduced the frequency of CD4+Foxp3+ or CD4+CD25+ Tregs in both the liver and spleen of HBV-carrier mice (Fig. 6A, 6B). We supposed that the reduced Treg frequency was due to the inhibition of Treg induction. Indeed, the Helios− cells among the Foxp3+ Tregs, which appeared to be the induced Tregs (26, 27), were reduced after IL-12–based vaccination therapy (Fig. 6C). In addition, CD4+CD25+ T cells from HBV-carrier mice treated with IL-12–based vaccination therapy were refractory to TGF-β–induced Foxp3 expression in vitro (Fig. 6D); moreover, we also found that these CD4+CD25+ effector T cells proliferated to a greater extent than cells from vaccine-treated control mice in the presence of Tregs (Fig. 6E), indicating that IL-12–based vaccination therapy induced effector T cells to overcome Treg-mediated inhibition. Consistent with these findings, splenocytes isolated from HBV-carrier mice completely inhibited HBsAg-specific proliferation of splenocytes from HBsAg-vaccinated naive mice in vitro, whereas splenocytes from IL-12–based vaccination-treated HBV-carrier mice did not inhibit the Ag-specific proliferation of these cells (Fig. 6F). These results suggest that IL-12–based vaccination therapy diminishes the frequency of Ag-specific inhibitory cells that normally exist in HBV-carrier mice.

**IL-12–based vaccination therapy induced robust hepatic HBV-specific CD8+ T cell responses**

A robust HBV-specific CD8+ T cell response has previously been reported to be critical for HBV elimination in the clinic (3). In our study, HBV-specific CD8+ T cells actually responded more quickly and robustly than CD4+ T cells. HBV-carrier mice receiving IL-12–based vaccination therapy showed rapidly increased CD11a hi CD8+ T cell responses, mainly because of the selective expansion of HBV-specific CD8+ T cells (Fig. 5C). Furthermore, HBV-specific CD8+ T cells from mice treated with IL-12–based vaccination therapy expressed higher KLRG1 and lower CD62L levels (Fig. 5D, 5E), a phenotype representing effector/memory T cells (25). In line with this phenotype, splenic HBV-specific CD8+ T cell effector function, assessed by IFN-γ and TNF-α secretion, was greatly enhanced after IL-12–based vaccination therapy compared with HBV vaccine alone or untreated controls (Fig. 5F, 5G). Specifically, both HBCAg- and HBsAg-specific IFN-γ–secreting CD8+ T cells were greatly increased after IL-12–based vaccination therapy (Fig. 5H, 5I), indicating that a multispecific and robust anti-HBV CD8+ T cell response was recovered. Taken together, these data showed that IL-12–based vaccination therapy could reinvigorate systemic HBV-specific CD4+ T cell responses in HBV-tolerant mice.

**IL-12–based vaccination therapy inhibited Treg generation**

The balance between effector/memory CD4+ and regulatory CD4+ T cells may be critical for the outcome of immune responses. As expected, IL-12–based vaccination therapy reduced the frequency of CD4+Foxp3+ or CD4+CD25+ Tregs in both the liver and spleen of HBV-carrier mice (Fig. 6A, 6B). We supposed that the reduced Treg frequency was due to the inhibition of Treg induction. Indeed, the Helios− cells among the Foxp3+ Tregs, which appeared to be the induced Tregs (26, 27), were reduced after IL-12–based vaccination therapy (Fig. 6C). In addition, CD4+CD25+ T cells from HBV-carrier mice treated with IL-12–based vaccination therapy were refractory to TGF-β–induced Foxp3 expression in vitro (Fig. 6D); moreover, we also found that these CD4+CD25+ effector T cells proliferated to a greater extent than cells from vaccine-treated control mice in the presence of Tregs (Fig. 6E), indicating that IL-12–based vaccination therapy induced effector T cells to overcome Treg-mediated inhibition. Consistent with these findings, splenocytes isolated from HBV-carrier mice completely inhibited HBsAg-specific proliferation of splenocytes from HBsAg-vaccinated naive mice in vitro, whereas splenocytes from IL-12–based vaccination-treated HBV-carrier mice did not inhibit the Ag-specific proliferation of these cells (Fig. 6F). These results suggest that IL-12–based vaccination therapy diminishes the frequency of Ag-specific inhibitory cells that normally exist in HBV-carrier mice.

**IL-12–based vaccination therapy induced robust hepatic HBV-specific CD8+ T cell responses**

A robust HBV-specific CD8+ T cell response has previously been reported to be critical for HBV elimination in the clinic (3). In our study, HBV-specific CD8+ T cells actually responded more quickly and robustly than CD4+ T cells. HBV-carrier mice receiving IL-12–based vaccination therapy showed rapidly increased CD11a hi CD8+ T cell responses, mainly because of the selective expansion of HBV-specific CD8+ T cells (Fig. 5C). Furthermore, HBV-specific CD8+ T cells from mice treated with IL-12–based vaccination therapy expressed higher KLRG1 and lower CD62L levels (Fig. 5D, 5E), a phenotype representing effector/memory T cells (25). In line with this phenotype, splenic HBV-specific CD8+ T cell effector function, assessed by IFN-γ and TNF-α secretion, was greatly enhanced after IL-12–based vaccination therapy compared with HBV vaccine alone or untreated controls (Fig. 5F, 5G). Specifically, both HBCAg- and HBsAg-specific IFN-γ–secreting CD8+ T cells were greatly increased after IL-12–based vaccination therapy (Fig. 5H, 5I), indicating that a multispecific and robust anti-HBV CD8+ T cell response was recovered. Taken together, these data showed that IL-12–based vaccination therapy could reinvigorate systemic HBV-specific CD4+ T cell responses in HBV-tolerant mice.
CD8αloCD8α+ T cells in PBL, liver, and spleen, reaching a peak at week 3 (Fig. 7A, 7B), which coincided with a rapid decline in HBV (Fig. 1). Among these HBV-specific effector/memory cells, both HBsAg-specific (208–216) and HBeAg-specific (93–100) CD8α+ T cells were dramatically increased after IL-12–based vaccination therapy (Fig. 7C, 7D). Moreover, similar to CD4+ T cells (Fig. 5), IL-12–based vaccination therapy also selectively induced proliferation (Fig. 7E) and activation of HBV-specific CD11a+CD80+CD8α+CD8α+ T cells rather than nonspecific CD8α+ T cells (Fig. 7F, 7G). Interestingly, this CD8α+ T cell stimulation effect only partially relied on IL-12, because HBV-carrier mice treated with IL-12 alone exhibited a lower increase of HBV-specific effector/memory CD8α+ T cells than those treated with IL-12–based vaccination therapy (Supplemental Fig. 3C–E). This result also indicated that a synergistic effect of IL-12 and HBsAg vaccine in stimulating HBV-specific CTL cells. Notably, IL-12–based vaccination therapy triggered IFN-γ secretion from both hepatic HBV-specific and non–HBV-specific CD8α+ T cells (data not shown), resulting in an overall 5- to 8-fold increase of hepatic IFN-γ–secreting CTL cells over HBV vaccine alone (Fig. 7H). Given that CTLs are the major effector cells for HBV clearance through IFN-γ–mediated noncytotoxic effects (28, 29), we speculated that the robust hepatic CD8α+ T cell responses induced by IL-12–based vaccination therapy dramatically reduced HBV titers, which could be beneficial for reversing systemic tolerance and eradicating HBV.

Discussion

Liver-induced systemic tolerance upon chronic infection poses a great challenge to the development of therapeutic vaccine, especially for CHB, which affects >350 million people around the world. In previous studies, several vaccination strategies attempted to break systemic HBV tolerance in an HBV-tg mouse model (8, 14, 30, 31); however, because HBV DNA integrates into the genome of mouse embryos in this model, their immune systems become nonresponsive (i.e., tolerant) to HBV because of central clonal deletion of HBV-specific T and B cells, which does not reflect the acquired tolerant state in most human CHB patients. Because HBV is unable to infect new hepatocytes in our HBV-carrier mouse model, no immunopathology or innate immune signals can occur, and low HBV titers are observed in HBV-carrier mice; these aspects of the model more closely resemble human asymptomatic chronic HBV carriers, who usually do not require treatment, than CHB patients (2, 3). However, the Ag-specific systemic tolerant state is similar in both human HBV carriers and our HBV-carrier mice, which is mainly induced by persistent HBV expression in liver and mediated by the “liver tolerance effects,” such as induction of Ag-specific regulatory cells, peripheral T cell clonal deletion, and anergy (32). Thus, our findings that IL-12–based vaccination therapy can reverse liver-induced acquired HBV tolerance may be instructive for therapeutic vaccine design against CHB carriers.

Although IL-12 monotherapy can significantly reduce HBV in HBV-carrier mice, these mice still persistently express HBV after IL-12 withdrawal and fail to rapidly eradicate virus upon “recarrying” HBV (Fig. 2A–C, Supplemental Fig. 3A, 3B). These data suggest that the therapeutic effect of IL-12 monotherapy mainly relies on the transient and nonspecific viral inhibition effect of IL-12 (18) rather than on triggering long-term anti-HBV–specific immunity through reversing intrinsic immune tolerance. In contrast with IL-12 monotherapy, vaccination therapy was originally de-

**FIGURE 7.** IL-12–based vaccination therapy induced robust hepatic HBV-specific CD8α+ T cell responses in HBV-carrier mice. HBV-carrier mice were treated with IL-12–based vaccination therapy, vaccine alone, or no treatment. Three weeks later, hepatic mononuclear cells and splenocytes were harvested. (A) CD11a+CD8α+ cell frequency among CD8α+ T cells in PBL was monitored weekly. (B) CD11a+CD8α+ cells were identified in liver (left) and spleen (right), gated on CD8α+ T cells. The frequencies of (C) HBeAg- and (D) HBsAg–specific CD8α+ T cells among splenic CD19–NK1.1–CD3–CD8α+ cells were determined by staining with HBeAg or HBsAg peptide–coupled H2k pentamers. (E) K67 staining of splenic CD11a+CD8α+ cells (right, HBV-specific) and CD11a+CD8α+ cells (left, non–HBV-specific) is shown. (F) KLRG1 and (G) CD62L staining of splenic CD11a+CD8α+CD8α+ T cells (HBV-specific) are shown. (H) Hepatic lymphocytes were stimulated with PMA and ionomycin in vitro for 4 h in the presence of monensin, and intracellular IFN-γ was examined by flow cytometry. The numbers of total IFN-γ+ hepatic CD8α+ T cells were calculated. (A) Data are shown as the mean ± SEM. (B–H) Each plot represents one mouse. Experiments were repeated three times with similar results. *p < 0.05, **p < 0.01, ***p < 0.001.
signed to induce long-term Ag-specific immunity to completely control the virus, but vaccine monotherapy was inefficient in HBV-carrier mice because it was not strong enough to reverse the systemic tolerance already induced in the host by HBV. However, during IL-12–based vaccination therapy, a series of host factors altered by IL-12, such as reduced viral titers, extensive immune activation, and proinflammatory cytokine milieu, facilitated the vaccine to become more effective in mounting robust anti-HBV immunity by reversing tolerance, subsequently leading to the elimination of HBV from the host. Notably, although a previous study showed that a DNA vaccine containing a mutant IL-12 and HBV expression plasmids could induce long-term HBV-specific CD8+ effector/memory cells in some lamivudine-treated CHB patients, the effect of IL-12 in this study was obscure (33), because serum IL-12 and IFN-γ levels were undetectable during IL-12/HBV vaccine combination therapy (33). Thus, our study provides more mechanistic insights into how the IL-12 protein functions synergistically with the vaccine to break the acquired tolerance state in HBV carriers. Interestingly, even in HBV-tg mice that had central tolerance toward HBV, IL-12–based vaccination therapy could still induce more anti-HBs production and further reduce HBV DNA than vaccine or IL-12 alone (data not shown), further demonstrating the unexpected effect of IL-12 in reversing T cell tolerance when used in combination with vaccine.

An essential role of IL-12 in our model is the induction of robust T cell responses, as our experimental evidence shows that both HBV-carrier Rag1−/− mice and HBV-carrier CD4−/− mice cannot eradicate HBV after IL-12–based vaccination therapy (Fig. 4C, 4D), and that hepatic HBV-specific CD8+ T cell responses are dramatically enhanced during this therapy (Fig. 7, Supplemental Fig. 3D, 3E). These results are consistent with previous reports demonstrating that CD8+ T cells are the main effectors of IFN-γ-mediated noncytolytic effects during HBV clearance in both mouse models and HBV-infected chimpanzees (28, 29). HBV-specific CD4+ T cells were functionally impaired in HBV-carrier mice (Z. Zeng, H. Wei, R. Sun, and Z. Tian, unpublished observations), suggesting that systemic HBV-specific CD4+ Th responses might need to be recovered to restore responses to HBsAg vaccination in HBV carriers. Indeed, both the number and effector function of HBV-specific CD4+ T cells were significantly augmented during IL-12–based vaccination therapy (Fig. 5). This strong T cell stimulation capacity of IL-12 could be beneficial for reversing the liver-induced clonal deletion/anergy of Ag-specific T cells. In addition, this therapy reduced not only hepatic (34), but also splenic CD4+Foxp3+ Treg frequency (Fig. 6), which was previously shown to partially contribute to HBV-induced tolerance within CHB patients and asymptomatic carriers (35, 36). Meanwhile, in accordance with a previous in vitro study (37), CD4+ T cells from mice treated with IL-12–based vaccination therapy seemed to be more resistant to Treg-mediated suppression, perhaps providing another mechanism for the efficient reversal of immune tolerance by IL-12–based vaccination therapy.

We speculate that the IL-12–induced cytokine milieu at a peripheral site could also be beneficial for reversing tolerance. On one hand, IL-12–induced Th1 cytokines, including high IFN-γ and TNF-α levels (data not shown), might overcome suppression mediated by inhibitory cytokines, such as IL-10 and TGF-β. On the other hand, IL-12 therapy also induced significantly increased levels of serum IL-6, a cytokine essential for follicular Th cell differentiation in mice (38) and viral control during chronic LCMV infection (39), especially in the later phase of therapy (e.g., at week 3, data not shown), which likely promoted Ab production in response to peripheral vaccination. Recent studies show that continuous strong IL-12 stimulation results in functional exhaustion of Ag-specific T cells, which exhibit upregulated Tim3, PD1, Lag3, and 2B4 expression, as well as downregulated effector molecule expression (40). This could be the fundamental reason to explain the failure of IL-12 monotherapy in clinic. However, in our model, IL-12 was administered at a relatively low dose for breaking tolerance to peripheral HBsAg vaccination, and none of the earlier-mentioned inhibitory receptors, except 2B4, were up-regulated on HBV-specific T cells during IL-12 therapy (data not shown), although the functional importance of this 2B4 expression on T cells remains to be defined. Therefore, we propose that using IL-12 to “help” rather than “kill” T cell responses may be a more rational and efficient use of IL-12 when applying IL-12–based immunotherapy.

Overall, our results demonstrate that the synergistic effect of IL-12 and HBV vaccine can overcome liver-induced systemic immune tolerance in HBV-carrier mice, leading to HBV clearance. Thus, this approach might be a promising immunotherapeutic strategy for CHB therapy. Furthermore, to our knowledge, this is the first study to explore the role of IL-12 in breaking hepatic Ag-induced systemic immune tolerance, and the therapeutic implications for this role of IL-12 can be extended to provide immunotherapy for other hepatic chronic infections or tumors when combined with a vaccine.

Disclosures
The authors have no financial conflicts of interest.

References


