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博士論文

Graduate Institute of Immunology
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Doctoral Dissertation

以 B 型肝炎病毒小鼠模式探討核心蛋白引發免疫反應之機制

Induction of Immune Response by Hepatitis B virus Core Protein in a Mouse Model

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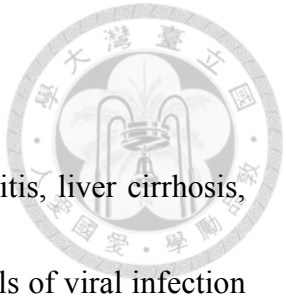
中文摘要

B 型肝炎持續性感染會造成慢性肝炎，肝纖維化和肝癌。近來在動物模式中研究病毒感染的報告指出淋巴球上的抑制性受體 (PD-1) 和配體 (PD-L1) 間的相互作用對於抑制 T 細胞的活性扮演重要角色。我們利用最近發展的 B 型肝炎小鼠模式來探討 T 細胞 exhaustion 現象及阻斷 PD-1/PD-L1 作用後對於肝浸潤 T 細胞的效應。在此小鼠模式中，我們證明了 B 型肝炎的持續性和肝浸潤 T 細胞的 PD-1 表現成正相關，在帶原小鼠中肝浸潤 CD8 T 細胞表現較高的 PD-1 和較低的 CD127 exhaustion 現象，並且利用單株抗體阻斷 PD-1/PD-L1 作用可增加病毒核心蛋白專一性的 IFN-γ 產生，而且可以反轉 T 淋巴球的 exhaustion 及增加小鼠對病毒的清除能力。我們的結果指出，在小鼠模式中阻斷 PD-1 的途徑可恢復對 B 肝病毒的免疫反應及增進對病毒的清除。

先天與後天免疫反應的調和才能有效的控制病毒感染，為了瞭解對抗 B 肝病毒的先天免疫反應，我們使用了一系列與先天免疫反應相關受體之基因缺陷小鼠來測試，結果發現除了腫瘤壞死因子缺失的小鼠外，其餘包括 IFN-α/β receptor, RIG-I, MDA5, MYD88, NLRP3, ASC, 和 IL-1R 基因缺失小鼠都與控制組相當。我們進一步發現，給予小鼠腫瘤壞死因子受體阻斷劑可促使 CD8 T 細胞 PD-1 表現、肝臟中病毒複製、核心抗原和表面抗原及血清病毒量增加；純化後的病毒核心蛋白可在小鼠引發腫瘤壞死因子及 IL-6 表現，但 IFN-β 則否。我們也發現病毒核心蛋白造成肝臟中白血球引發的腫瘤壞死因子是清除病毒的重要途徑，這些結果提供了腫瘤壞死因子相關的先天免疫反應對抗病毒的機制，也解釋了臨床上使用腫瘤壞死因子阻斷劑造成 B 肝病毒再活化的機制。

關鍵字：

B 型肝炎病毒，PD-1 阻斷，病毒持續性，先天免疫力，腫瘤壞死因子



Abstract

Persistent hepatitis B viral (HBV) infection results in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Recent studies in animal models of viral infection indicate that the interaction between the inhibitory receptor, programmed death (PD)-1, on lymphocytes and its ligand (PD-L1) play a critical role in T-cell exhaustion by inducing T-cell inactivation. We studied T-cell exhaustion and the effects of PD-1 and PD-L1 blockade on intrahepatic infiltrating T-cells in our recently developed mouse model of HBV persistence. In this mouse animal model, we demonstrated that the chronicity of hepatitis B virus infection was associated with PD-1 expression on intrahepatic lymphocytes. The Intrahepatic $CD8^+$ T-cells expressed higher levels of PD-1 and lower levels of CD127 in carrier mice. Blockade of PD-1/PD-L1 interactions by an anti-PD-1 monoclonal antibody (mAb) increased HBcAg-specific interferon (IFN)-γ production in intrahepatic T lymphocytes and reversed the exhausted phenotype in intrahepatic T lymphocytes and viral persistence to clearance of HBV *in vivo*. Our results indicated that PD-1 blockage reverses immune dysfunction and viral persistence of HBV infection in a mouse animal model, suggesting that the anti-PD-1 mAb might be a good therapeutic candidate for chronic HBV infection.

An efficient control of virus infections requires the coordinated actions of both innate and adaptive immune responses. In order to define the role of innate immunity effectors

against HBV, viral clearance was studied in a panel of immunodeficient mouse strains. Our results demonstrate that HBV viral clearance is not changed in IFN-α/β receptor (IFNAR), RIG-I, MDA5, MYD88, NLRP3, ASC, and IL-1R knockout mice. In contrast, HBV persists in the absence of tumor necrosis factor-alpha (TNF-α) or in mice treated with the soluble TNF receptor blocker, Etanercept. In these mice, there was an increase in PD-1-expressing $CD8^+$ T-cells and an increase of serum HBV DNA, HBV core, and surface antigen expression as well as viral replication within the liver. Furthermore, the induction of TNF-α in clearing HBV is dependent on the HBV core, and TNF blockage eliminated HBV core-induced viral clearance effects. Finally, the intra-hepatic leukocytes (IHLs), but not the hepatocytes, are the cell source responsible for TNF-α production induced by HBcAg. These results provide evidences for TNF-α mediated innate immune mechanisms in HBV clearance and explain the mechanism of HBV reactivation during therapy with TNF blockage agents.

Key words: Hepatitis B virus; PD-1 blockage; viral persistence; innate immunity; Tumor necrosis factor

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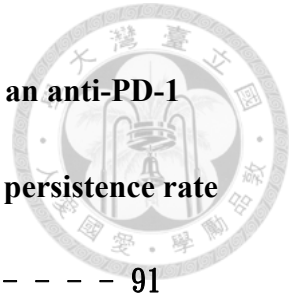


Abbreviations

HBV: Hepatitis B virus

SPF: specific pathogen-free

NTCP: sodium taurocholate cotransporting polypeptide

HCV: hepatitis C virus

NPC: nuclear pore complex

HIV: human immunodeficiency virus

cccDNA: covalently closed circular DNA

IFN: interferon

pgRNA: pregenomic RNA

TNF-α: tumor necrosis factor-α

ALT: alanine aminotransferase

HDI: hydrodynamic injection

PD-1: programmed death-1

NLRP3: Nod-like receptor family protein 3

PBMC: peripheral blood mononuclear cells

LCMV: lymphocytic choriomeningitis virus

Treg: regulatory T cells

RIG-I: retinoic acid-inducible gene 1

IHL: intra-hepatic leukocyte

RLR: RIG-I like receptor

IRF3: interferon regulatory factor 3

LSEC: liver sinusoidal endothelial cell

NLR: NOD-like receptor

CTL: cytotoxic T lymphocyte

mAb: monoclonal antibody

IPS-1: interferon-beta promoter stimulator 1

JAK: Janus kinase

FIX: coagulation factor IX

IFNAR: IFN-α/β receptor

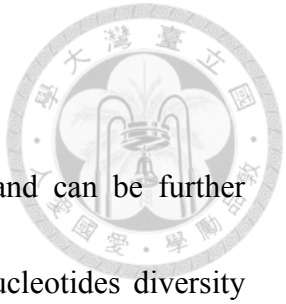
MDA5: Melanoma Differentiation-Associated protein 5

C4BP: C4-binding protein

HSPG: heparan sulfate proteoglycans

LPR: lipoprotein receptor-related protein

Chapter I
Introduction



1.1 Virology of Hepatitis B virus

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family and can be further classified into eight major genotypes (A to H) according to the nucleotides diversity of >8% (Stuyver et al., 2000). The HBV contains a partially double-stranded 3.2 kb DNA genome and exhibits very limited host range and simply infects humans and chimpanzees naturally. The entry of HBV into cell is mediated by its envelope proteins. Most studies have shown that the pre-S1 domain of large envelope protein is required for receptor(s) binding and infection of hepatocytes (Blanchet and Sureau, 2007; Le Duff et al., 2009). Recently, a multiple transmembrane transporter sodium taurocholate cotransporting polypeptide (NTCP) is identified as a functional receptor for HBV (Yan et al., 2012). Interaction of pre-S1 region and NTCP allows HBV entrance to hepatocytes. Once the virus is inside a hepatocyte, HBV starts to uncoat and nucleocapsid is transported to the nuclear pore complex (NPC), then viral cores disassemble, and genomic DNA is transferred to the nucleus (Kann et al., 1997). Inside the nucleus, the viral DNA is converted to the covalently closed circular DNA (cccDNA). The cccDNA serves as the template for generation of viral transcripts (Tuttleman et al., 1986). Four RNA species: 3.5-, 2.4-, 2.1- and 0.7- kb are transcribed and exported to the cytoplasm where the viral genes are translated. The viral polymerase (pol), core antigen (HBcAg) and e antigen (HBeAg) are encoded from the longest open

reading frame. Expression of viral envelope proteins including large, middle and small surface antigen (HBsAg) are translated from 2.4- and 2.1 kb transcripts. The HBV X protein (HBx) is generated from 0.7-kb mRNA and acts as a transactivator with multi-functions including viral replication and spread of virus (Bouchard and Schneider, 2004; Zoulim et al., 1994). In addition to serving as messenger RNA (mRNA) for the nucleocapsid and Pol proteins, the 3.5-kb pregenomic RNA (pgRNA) also acts as the template for reverse transcription of the viral genome. Reverse transcription occurs within the capsid particle, which also co-packages cellular proteins such as heat shock protein 70, 90 and kinase (s). The cytosolic DNA-containing nucleocapsid can transport either to the nucleus or to the ER membrane, where the envelope proteins assemble around the nucleocapsids to form mature virions (Kann et al., 1999; Rabe et al., 2003).

1.2 Natural history of Hepatitis B virus infection

Hepatitis B virus is the major etiology of human liver diseases worldwide. Infection by this virus can cause both acute and chronic liver disease. Around 2 billion people are infected, and 240 million people become chronic carriers, who have virus persistently for more than 6 months. The resolution of primary HBV infections is age-dependent. Most of infants infected during the age of one year develop chronic infections. However, only less than 5% of adults who acquire infection become HBV chronic carriers, but up

to 30% of carriers develop progressive liver disease such as chronic hepatitis, cirrhosis and hepatocellular carcinoma and claim 600000 people die every year (Roberts and Gores, 2005). Young children with primary HBV infection are often asymptomatic and predispose to become chronic carriers. In contrast, most primary infections occur in adults are self-limited with elimination of virus from liver and peripheral blood and develop protective immunity towards HBV (Hoofnagle, 1981; Wright and Lau, 1993). In primary HBV infection, serum level of HBsAg becomes detectable within an incubation period of 4-10 weeks, followed by production of anti-HBc antibodies, which are mainly of IgM isotype (Hoofnagle, 1981). The viral titers are always up to 10^9 to 10^{10} virions per milliliter in acute infection (Ribeiro et al., 2002). In most cases, the blood level of HBeAg is detectable and the appearance of circulating HBeAg is correlated well with the frequency of infected hepatocytes in animal studies (Kajino et al., 1994). Serum levels of alanine aminotransferase (ALT) reflect liver injury. Once T cell–mediated immune response that triggers liver injury is mounted, ALT levels are elevated, and titers of virus in blood and liver begin to decrease. Thus, the virus can be eradicated from hepatocytes without massive liver destruction. The HBsAg and HBeAg are undetectable in the circulation, and anti-HBs antibodies become evident.

In persistent HBV infection, circulating HBsAg is continuously detectable in peripheral blood and virus production persistently, often for life. However, the viral titers in

chronic HBV infection are generally lower than during primary infection. HBeAg positivity often reflects high levels of viremia that are typically 10^7 to 10^9 virions per milliliter and are highly infectious (Weinberger et al., 2000). But carriers with anti-HBe antibodies appearance generally have lower levels of viremia. The natural course of chronic HBV infection is dynamic. Nevertheless, HBeAg tend to decline from the circulation, along with seroconversion to anti-HBe antibodies- positive carriers with the passage of time that occurs at an estimated rate of 5-10% per year in HBV carriers (Ribeiro et al., 2002). Seroconversion to anti-HBe antibodies may accompany the reduction of viral titers five orders of magnitude (Tedder et al., 2002). The disappearance of HBeAg is often accompanied by elevation of ALT levels, so called a flare, a phenomenon which suggests that the immune-mediated reactions destroy the infected-hepatocytes. Thus, the natural history of HBV chronic infection implies that there exists continuing immune attack on infected hepatocytes, but inadequate to clear infection.

1.3 Immune-mediated pathogenesis of Hepatitis B

1.3.1 Adaptive immunity against HBV

Effective HBV clearance requires initial antigen encounter by innate immune response, which subsequently activates the cellular inflammatory response. Appearance of

HBV-specific CD4- and CD8-mediated response generally after the robust increase in HBV replication at the early phase of infection (Fisicaro et al., 2009; Webster et al., 2000). These response are much stronger than those observed in chronic infection (Bertoletti et al., 2010). In self-limited infection, viral titers in blood decline more than 90% rapidly (usually within 2-3 weeks) after the peak of viral replication. The liver damage is also detectable, suggesting that removal of virus by non-cytopathic mechanism occurs (Guidotti et al., 1999). Intrahepatic HBV-specific cytotoxic T cells are recruited by chemokines secretion (such as CXCL10) and by platelet activation (Iannacone et al., 2005; Sitia et al., 2007). Following control of infection, elevated expression of CD127 and decrease in programmed death-1 (PD-1) on HBV-specific $CD8^+$ T cells are observed (Boettler et al., 2006). In persistent HBV infection, it has been found that the adaptive immune system exerts active immune suppression which inhibits HBV clearance and promotes the development of immune tolerance. An increasing number of $CD4^+CD25^+$ regulatory T cells are found in the peripheral blood and livers of chronic hepatitis B patients. The HBV Ag-specific IFN-γ production and proliferation of peripheral blood mononuclear cells (PBMC) are upregulated when depletion of regulatory T cells from culture (Xu et al., 2006). Furthermore, inhibition of HBV-specific CTL response and IFN- secretion have been found to be exerted by PD-L1/PD-1 interaction in the liver (Fisicaro et al.; Maier et al., 2007; Peng et al.,

2008a). In chronic infection, HBV persistence is always associated with dysfunction of HBV-specific CD4 and CD8 T cells. The strength of T cell response usually is inversely correlated to viral loads (Boni et al., 2007; Maini et al., 2000). Thus, in patients with highly titers of viral DNA, the HBV-specific T cell response is more suppressed (Fisicaro et al., 2010). The defect of T cell function is probably due to long-term exposure of T cells to highly concentration of viral antigens and tolerogenic microenvironment of liver (Crispe, 2011; Schildberg et al., 2011).

In spite of the importance of T cells producing Th1 cytokines (such as IL-2, IL-12 and IFN-γ) on the control of HBV infection, other T cell subsets, like regulatory T cells (Treg), Th17, also play the roles in the immune reactions upon HBV infection. Many studies have shown that the highly frequencies of Treg, Th17 and IL-22 producing cells are observed in patients with chronic hepatitis than in healthy individuals (Xiang et al., 2012; Xu et al., 2006; Zhang et al., 2010). CXCR6 receptors recruit IL-17- and IL-22-producing T cell populations to intrahepatic environment, but these correlations do not define the effects of such T cells on the pathogenesis of HBV infection (Billerbeck et al., 2010). Th17 cells play important roles in the pathogenesis of autoimmune and several inflammatory diseases. They are also observed in highly frequency in HBV carriers with severe liver damage (Zhang et al., 2010). Nevertheless, IL-17-producing HBV-specific T cells are undetectable in both liver and peripheral

blood in acute and chronic HBV patients (Gehring et al., 2011). The significance of expression of IL-17 during HBV infection is still unclear.

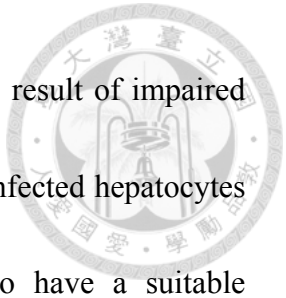
1.3.2 Innate immunity against HBV

Innate receptors recognize pathogens or pathogen-derived products in different cellular compartments, such as the plasma membrane, the endosomes or the cytoplasm, and induce the expression of cytokines, chemokines and co-stimulatory molecules to eliminate pathogens and shape pathogen-specific adaptive immune responses. In the case of HBV infection, the innate immune reactions are still obscure. Induction of type I interferons and pro-inflammatory cytokines are often undetectable during the initial phase of infection (Dunn et al., 2009; Stacey et al., 2009). It raises the possibilities that HBV may escape innate recognition or HBV suppresses innate immunity. However, the vigorous cytotoxic T lymphocytes activation is induced in acute HBV infection. It is believed that there exists innate sensors to recognize HBV to induce immune response towards HBV. Indeed, HBV polymerase is shown to interact with DDX3 to block TLR3 or RIG-I-mediated IFN-β production (Wang and Ryu, 2010; Yu et al., 2010). HBV X protein can inhibit IFN-β production through interaction with IPS-1, which is the adaptor for RIG-I like receptors (RLR) (Kumar et al., 2011). Secretory HBV proteins

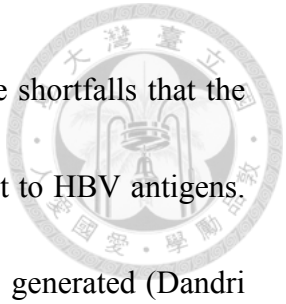
such as HBeAg and HBsAg have been shown to block TLR-mediated innate response (Wu et al., 2009).

Besides hepatocytes which represent the only target of infection, intrahepatic antigen presenting cells including DC, Kupffer cells and LSEC (Racanelli and Rehermann, 2006a, b) may also induce innate immune response against HBV. DC and Kupffer cells can take up HBV virions or subviral particles (Bauer et al., 2011a, b; Untergasser et al., 2006), and isolated human liver nonparenchymal cells up-regulate IL-1, IL-6, TNF-α and IFN-β gene expression level after contact to HBV virions rather than being infected (Hosel et al., 2009). NK cells also play critical roles in control of early phase of HBV infection (Fisicaro et al., 2009). Accumulating evidence suggests that HBV can regulate the expression of TLRs in patients with chronic hepatitis B (Barton, 2007). TLR2 expression is shown to be down-regulated in peripheral blood mononuclear cells (PBMCs), KCs, and hepatocytes of patients with chronic hepatitis B (Visvanathan et al., 2007). The data showing that PBMCs from patients with chronic hepatitis B expresses lower levels of TLR1, TLR2, TLR4, and TLR6 messenger RNA transcripts compared with those from healthy controls (Chen et al., 2008).

1.4 Animal models for exploring immune mechanism of hepatitis B



Although the chronicity of Hepatitis B virus (HBV) infection is the result of impaired HBV-specific immune responses that cannot eliminate or clear the infected hepatocytes efficiently, many issues remained unsettled. It is thus crucial to have a suitable laboratory animal to study the immunopathogenesis of HBV infection and the mechanisms of HBV persistence. However, the study of HBV infection has been hampered by the shortage of animal model susceptible to HBV infections (such as chimpanzees) and the inability of HBV to propagate in common experimental animals, namely, mouse. Nonetheless, the immunopathogenesis of HBV infection has been obtained from observation of human infections and was greatly enhanced by studies in chimpanzees, woodchuck, and HBV-transgenic (Tg) mice (Chisari et al., 1985; Moriyama et al., 1990; Rehermann and Nascimbeni, 2005). Chimpanzees seem to be an ideal model for HBV infection because this primate is the natural host for this virus. However, the high expense, restricted source and reagents for analysis and outbred genetic background limit their feasibility. Woodchucks infected with woodchuck hepatitis virus can develop similar disease pattern to that of human beings (Summers, 1981). This animal model are widely used to explore the life cycle of virus and therapeutic strategies (Roggendorf et al., 2007), but not suitable for studying immunopathogenesis because of the outbred genetic background. While the HBV-Tg mice serve an important role in delineating the mechanism of HBV clearance and



persistence (Chisari et al., 1985), the system nonetheless inherits the shortfalls that the mice produce and express HBV antigens but are congenially tolerant to HBV antigens. Recently, a human liver chimeric mouse model (uPA/SCID mice) is generated (Dandri et al., 2001). The transplanted human hepatocytes are repopulated to replace mice hepatocytes in these uPA/SCID mice and are susceptible to HBV infection. Another humanized mice to allow mass production of human hepatocytes are $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ mice (FRG mice), which is T-, B- and NK-cell deficient (Shafritz, 2007). Nevertheless, dysfunction of immune system in these mice makes it difficult to reflect the scenario of natural infection.

To meet the requirement of a mouse model resembling natural chronic HBV infection in human adult, there are several approaches in the development of mouse animal model by introducing HBV DNA into the mouse liver. Among them, there are three commonly used ones as hydrodynamic-based transfection of HBV DNA, delivery of adenovirus or adeno-associated viral vectors containing HBV DNA. These nontransgenic mouse animal models for HBV infection provide new approaches to investigate the mechanisms of HBV clearance and persistence.

1.4.1 Hydrodynamic-based transfection of HBV mouse model

Hydrodynamic injection is a simple and efficient method to deliver genetic materials into liver *in vivo* (Liu et al., 1999). High level of gene expression in liver is achieved by injection of large volume of DNA solution (7% of body weight) via mice tail vein within 5-8 seconds. This procedure results in high hydraulic pressure in the inferior vena cava and pushing the DNA into hepatic vein. A sharp increase in venous pressure enlarges the liver fenestrae and enhances the membrane permeability, allowing for fluid extravasation into parenchymal cells (Herweijer and Wolff, 2007; Zhang et al., 2004). The DNA internalization via this physical process is receptor-independent.

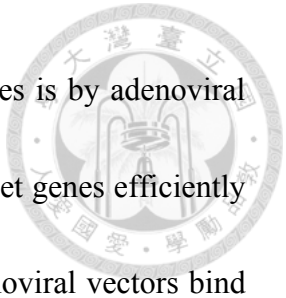
Immuno-competent mice receiving replication-competent HBV DNA injection hydrodynamiclly display acute self-limiting hepatitis with very different rates of clearance, whereas the persistent expressions of HBV antigens are observed in hepatocytes of immunocompromised mice (Yang et al., 2002). These results suggest that host immune response against viral transgene products may contribute to viral DNA clearance. Indeed, cellular immune responses are elicited in immune-competent mice receiving hydrodynamiclly delivery of HBV DNA (Huang et al., 2006). The vectors backbone to carry the viral transgenes seems to be a factor to influence HBV clearance in *in vivo* transfection models. It has been shown that excision of covalent linkage of bacteria DNA helps to increase maintenance of the transgenes in mouse liver (Riu et al., 2005). It is likely that the unknown sequence in AAV vector backbone

regulates long-term expression of HBV transgenes and leads to persistent HBV surface antigenemia. The genetic background of recipient mice also determine viral clearance (Huang et al., 2006). It is known that HBV-specific cytotoxic T cell response is the critical factor mediating HBV clearance. It is plausible that different HBV-CTL response occurs in various mice strains. Among certain mouse strains, persistence of HBV DNA in mice liver is accompanied by few activated intrahepatic cytotoxic T cells (Chen et al., 2012). Restoration of T-cell function by blockade of T-cell inhibitory receptor, PD-1, in HBV-carrier mice enhances viral clearance and reduces viral persistence (Tzeng et al., 2012). Recently, the immune effectors that involved in removal of HBV DNA in hydrodynamically transfected mice model are explored (Yang et al., 2010). The $CD4^+$ and $CD8^+$ T cells play the major roles in viral clearance. Interestingly, the innate immune effectors such as NK cells, type I interferon or TNF-α-mediated pathways are also critical for elimination of HBV DNA. Deficiency of IFN-beta signaling delays the HBV elimination, however, the viral-induced IFN-beta production in the transfection model is still minimal. In contrast, HBV infection prevents induction of IFN-beta or activation of IFN-alpha signaling in HBV-infected primary human hepatocytes or in chimeric mice (Hosel et al., 2009; Lutgehetmann et al., 2011). In addition, IL-15 exhibits the anti-HBV function in the interferon beta-dependent manner, but is neither dependent on NK cells nor on the activity of T- or

B-cells (Yin et al., 2012). NK cells deficient mice fail to eliminate HBV DNA in mice liver, suggesting the essential role of NK cells in control of HBV in murine model (Yang et al., 2010).

Hepatitis B virus core antigen (HBcAg) is the critical factor to determine viral clearance in hydrodynamic-based *in vivo* transfection (Lin et al., 2010). Intriguingly, the HBcAg capsid structure seems to be the determinant to induce HBV-specific CTL response and production of antibodies against HBcAg or HBsAg, since the assembly-defective HBcAg mutant (HBcY132A) fails to induce detectable immune response (Lin et al., 2012). The regulatory protein X of hepatitis B (HBx) has been shown to support viral replication (Keasler et al., 2007) and involve in various cellular signaling pathways, including proliferation, DNA repair and transformation (Keng et al., 2011). HBx also targets to innate adaptor IPS-1 to suppress cellular IFN-beta production (Kumar et al., 2011). Administration of attenuation of HBV X gene expression by small interfering RNA containing 5'-end triphosphate inhibits HBV replication and decreases serum level of HBsAg in hydrodynamic transfected HBV carrier mice (Ebert et al., 2011; Han et al., 2011). In addition, the administration promotes the increased serum level of IFN-beta, suggesting the activation of innate receptor(s) is critical for antiviral activity.

1.4.2 HBV mouse model generated by delivery of adenovirus



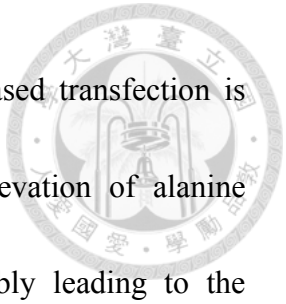
Another route adopted to deliver HBV genome into mice hepatocytes is by adenoviral vector. Adenoviral vectors are the excellent vehicles for transfer target genes efficiently into livers of immuno-competent mice (Bramson et al., 1995). Adenoviral vectors bind to coagulation factor IX (FIX) and complement component C4-binding protein (C4BP), and target to hepatocytes through cell surface heparan sulfate proteoglycans (HSPG) or low-density lipoprotein receptor-related protein (LPR) (Shayakhmetov et al., 2005). The receptor mediated genes delivery leads to infection of more than 90% hepatocytes (Hartman et al., 2007). Adenoviral infection induces upregulation of interferon-related genes such as MCP-1, IP-10, RANTES, MIP-2 etc. (Liu et al., 2003). Furthermore, the elevation of plasma cytokines and chemokines (e.g. KC, G-CSF, IL-6, IL-12 etc.) in adenovirus-infected livers are found to be MyD88-dependent (Hartman et al., 2007). Since the administration of empty adenoviral vectors induce innate immune response in mice liver, it is not surprising that high dose of HBV genome transfer via adenoviral vectors leads to HBV clearance, whereas persistent expression of HBV antigens results from low dose of adenoviral vectors delivery (Huang et al., 2012). Consistent with the HBV clearance in adenoviral-based transduction, the adaptive immune system, including HBV-specific CTL response and anti-HBs antibody production, is activated in high dose of adenoviral-HBV infection. Induction of sufficient B-cell response is accompanied by termination of HBV replication (von Freyend et al., 2011).

1.4.3 Adeno-associated viral vectors (AAV)-based HBV mouse model

Adeno-associated virus enter into target cells through interaction of viral capsid with HSPG in cell surface, and this binding is enhanced by integrins and growth factor receptors (Goncalves, 2005). While long-term expression of transgene within cells could be due to tolerance of humoral or cellular immune response inducing by AAV (Breous et al., 2010; Mingozzi et al., 2003). Recently, a novel HBV model in immuno-competent mice is generated by transfer of HBV genome using trans-splicing adeno-associated vectors (Huang et al., 2011). In this model, the production of HBV virions and proteins persist in liver and circulation for a long period of time, and this phenomenon is independent on mice genetic background. The profiles of viral antigen and antibodies in mice are similar to that clinic observed in human. More interestingly, the AAV/HBV- transduced mice could develop hepatic tumors (adenoma or hepatocellular carcinoma) (Huang et al., 2011).

1.4.4 Comparison of the advantages and disadvantages in each model

Delivery of HBV genome into C57BL/6 mice liver by hydrodynamic injection leads to rapid clearance of viral DNA template (Chen et al., 2012; Yang et al., 2002; Yang et al., 2010). However, by this technique, long-term maintenance of HBV transgenes in mice liver is also observed by using different vector (Huang et al., 2006), suggesting that



persistence of genetic materials in mice liver by hydrodynamic-based transfection is plasmid backbone-dependent. Hydrodynamic injection induces elevation of alanine aminotransferase level and hepatocytes necrosis, which is probably leading to the induction of proinflammatory cytokines (Racz et al., 2011). Adenoviral infection also induces immune activation when targets to liver (Hartman et al., 2008). In contrast, infection of adeno-associated vectors results in inactivation of immune response and maintain the persistence of transgenes (Breous et al., 2009).

Several HBV mice models have been generated in immune-competent mice background by different strategies of viral DNA transfer. The adenoviral vectors-mediated HBV genome transfer targets more than 90% of hepatocytes (Huang et al., 2012), whereas hydrodynamic transfection is approximately 10% of hepatocytes delivery (Yang et al., 2010). Accordingly, the activation of HBc-specific CTL response and anti-HBs antibody production are associated with HBV clearance in each model. The characteristics of each mice animal model is listed in Table 1.

HBV transgenic mice are useful tool to investigate viral biology and to screen therapeutic drugs that inhibit HBV replication. However, the immunopathogenesis of hepatitis in HBV-Tg mice are only observed by transfer of cytotoxic T lymphocytes because of tolerance of adaptive immunity to viral antigens (Moriyama et al., 1990). Thus, the limitation of HBV-Tg mice to explore innate and adaptive immune response

toward HBV prompts the development of non-transgenic HBV mice model. Delivery of HBV genome into immuno-competent mice by adeno-associated virus, adenovirus or hydrodynamic-based transfection leads to efficient viral genes expression in liver. The host immune response against HBV are elicited by these transfer methods (Huang et al., 2012; Tzeng et al., 2012; Yang et al., 2002). The analysis of immune effectors involved in HBV clearance are available by using specific gene-deficient mice. However, the procedure-induced immune response may interfere the host immunity against HBV. The routes of viral genome delivery are different from that of natural infection.

Chapter II
Materials and Methods

2.1 Animals and HBV constructs

Male C57BL/6 and BALB/c mice were obtained from the Animal Center of National Taiwan University and maintained under specific pathogen-free (SPF) conditions. Interferon-α receptor (IFN-α R) knockout mice were kindly provided by Dr. Guann-Yi Yu (National Health Research Institute, Taiwan). TNF-α knockout mice were obtained from Dr. Chun-Jen Chen (National Taiwan University, Taiwan). MyD88 knockout mice were provided by Dr. Nien-Jung Chen (National Yang-Ming University, Taiwan). The ASC and NALP-3 knockout mice were obtained from Dr. Jenny Ting (University of North Carolina, NC). The RIG-I and MDA-5 knockout mice were gifts from Dr. Akira (Osaka University, Japan). All animal experiments were performed according to regulations approved by the Animal Ethical Committee of National Taiwan University. The HBV constructs and pAAV/HBV1.2 mutants were generated by site-directed mutagenesis as previously described (Huang et al., 2006; Lin et al., 2010). Briefly, site-directed mutagenesis was used to generate pAAV/HBV1.2 mutants with a QuickChang II site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Then, the mutants were amplified by a polymerase chain reaction (PCR) using pAAV/HBV1.2 as a template.

2.2 Hydrodynamic injection

C57BL/6 mice and BALB/c (male, 6~7 weeks old) were anesthetized with ketamine and xylazine. Ten micrograms of HBV plasmid DNA in phosphate-buffered saline (PBS) in a volume equivalent to 8% of the mouse body weight was injected via tail veins within 5 s. Plasmid DNA were purified by using EndoFree Maxi plasmid kit (Qiagen, Hilden, Germany). For trans-complementation assay, 10 μg of pAAV/core-null was combined injection with 10 μg of pFLAG-CMV2/HBc or pFLAG-CMV2/HBcY132A mutant (Lin et al., 2012). Serum HBsAg were assayed as indicated time points to monitor the state of HBV persistence.

2.3 Isolation of intrahepatic leukocytes

Mice were anesthetized with ketamine and xylazine. The chest and abdomen were opened. Liver was perfused with 10mL PBS and whole liver was excised, weighted, and put into a 70μm nylon mesh. Liver tissue was minced by a plunger and passed through 70μm nylon mesh and obtained liver cell suspension. The liver cell suspension was collected and washed with HBSS to final volume 50mL. Hepatocytes and large cell clumps were removed by 50xg centrifugation at 4℃ for two times, then the pellet was discarded. The supernatant containing intrahepatic leukocytes (IHLs) was pelleted by 300xg centrifugation at 4℃ for 10 min. Cell pellet was resuspended in 40% HBSS and layered upper 70% percoll (GE Healthcare, Piscataway, NJ) gently. Next, cell was

centrifuged at 1200xg for 20 minutes at 25℃. Viable IHLs should recover at 40% / 70% percoll interphase. IHLs were collected, washed with 15mL HBSS, and centrifuged at 300xg for 10 minutes at 4℃. Cell pellet was collected for the downstream applications.

2.4 Isolation of primary hepatocytes

Mice were sacrificed at the indicated time points, and the liver were perfused with 50mL calcium- and magnesium-free Hank's Balanced Salt Solution and 0.04% collagenase. The mice livers were collected and passed through 100μm nylon mesh. The liver cell pellets were harvested by centrifugation. The pellets were washed by hepatocyte wash buffer. The pellets then were resuspended with 45% percoll solution. The hepatocytes were collected from pellets and cultured for further applications.

2.5 Detection of the HBV antigen, antibody (Ab), and DNA

Serum levels of HBsAg, HBeAg, anti-HBc, and anti-HBs Abs were determined using the AXSYM system kit (Abbott Diagnostika, Wiesbaden, Germany). The cutoff value for determining HBsAg positivity was a signal-to-noise (S/N) ratio of ≥ 2 and a signal-to-cutoff (S/CO) ratio of ≥ 1. To detect serum HBV DNA, each serum sample was pretreated with 25 units of DNase I (*Roche* Diagnostics, Mannheim, Germany) at 37 °C overnight, and total DNA was extracted and HBV DNA was detected by a

real-time PCR as previously described (Huang et al., 2006; Lin et al., 2010). Serum alanine transferase (ALT) was measured on a TBA-200FR automated clinical chemistry analyzer (Toshiba, Tokyo, Japan) as previously described (Huang et al., 2006).

2.6 Liver tissue preparation and immunoblotting

Mice were anesthetized by intraperitoneal injection using ketamine and xylazine. Mice were then subjected to intracardiac perfusion with PBS prior to collection of liver tissue. The livers were exposed, removed, and minced into small pieces. For Southern blotting, Fifty milligrams of mouse liver tissue was lysed in 700 μl of DNA extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.5 mg of proteinase K) at 37°C overnight. After incubation, RNase A (20μg/ml) was added for further 1 h incubation at 37°C. Total DNA was purified by phenol and chloroform extraction and precipitated by isopropanol, and then dissolved in 0.1× TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) with 20 μg/ml of RNase A. Thirty microgram of total DNA from a mouse liver tissue was used for Southern blot analysis of HBV DNA. Mouse DNA was digested with 40 units of HindIII (NEB) at 37°C overnight before gel electrophoresis.

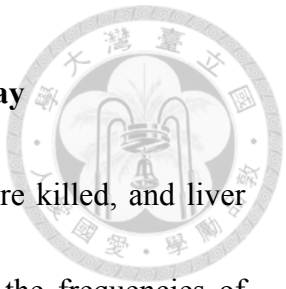
RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One milliliter of TRIzol was added to 50 mg of mouse liver tissue. Purified ten micrograms of RNA was used for Northern blotting. Both Southern

and Northern blot analyses were performed using digoxigenin (DIG)-labeled probes system. DIG-HBx and DIG–mouse glyceraldehyde-3-phosphate dehydrogenase (DIG-mGAPDH) probes were used to analyze HBV DNA/RNA and mouse GAPDH mRNA, respectively.

For Western blotting of HBcAg, liver extracts were prepared by lysing liver tissue in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.25% sodium deoxycholate, 0.1% SDS). Fifty microgram of protein lysates was separated by 15% SDS-PAGE, then transferred to PVDF membrane. The membrane was blotted by using rabbit anti-HBcAg (LTK BioLaboratories), mouse anti-β-actin (*Sigma-Aldrich, St. Louis*, MO), horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Promega, Madison, WI). The membrane was developed in an ECL system (Amersham Biosciences, Arlington Heights, IL).

2.7 Immunohistochemistry

The perfused liver samples were embedded in optimal cutting temperature compound (OCT). Intrahepatic HbcAg or HbsAg were detected by immunohistochemical staining with rabbit anti-HBc (Dako, Glostrup, Denmark) or anti-HBs antibodies (Biomeda, Foster City, CA) and Envision System, HRP (DAB) (Dako, Glostrup, Denmark). Hematoxylin was used to stain liver section nuclei.



2.8 Interferon (IFN)-γ enzyme-linked immunospot (ELISpot) assay

At indicated time points after the hydrodynamic injection, mice were killed, and liver mononuclear cells and splenocytes were cultured and assayed for the frequencies of antigen-specific IFN-γ- secreting cells using an ELISPOT kit (BD Biosciences, San Jose, CA). Briefly, 10^6 splenocytes or 10^5 liver mononuclear cells were co-cultured with 1 μg/ml of rHBcAg (ID Labs, London, Canada) in 200 μl RPMI 1640 supplemented with 10% fetal calf serum (FCS). Cell suspensions were incubated for 20 h. Spot-forming cells were revealed with a biotin-conjugated antibody, streptavidin-horseradish peroxidase (HRP) and AEC substrates (*Sigma-Aldrich*) and were analyzed using the ImmunoSpot series 5 analyzer (Cellular Technology Limited, Cleveland, OH)

2.9 Flow cytometry

For flow cytometry, allophycocyanin (APC)-conjugated anti-mouse CD3 (BD Biosciences, Palo Alto, CA), phycoerythrin (PE)-conjugated anti-mouse PD-1, fluorescein isothiocyanate (FITC)-conjugated anti-NK1.1 or anti-CD49b or anti-CD127 (eBioscience, San Diego, CA) and PE-Cy5.5-conjugated anti-mouse CD4 or CD8 (BD Biosciences Pharmingen) monoclonal (m)Abs were used for flow cytometry. The intracellular FoxP3 protein was stained using the cell-fixation/cell-permeabilization kit

included in the FoxP3 protein staining kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. For the flow cytometric analysis, 10^5 cells were labeled in a fluorescence-activated cell sorter (FACS) buffer (PBS/2% FCS/0.1% sodium azide), fixed in 1% paraformaldehyde (*Sigma-Aldrich, St. Louis*, MO) and analyzed on a FACSCalibur using the CellQuest software (Becton Dickinson, Mountain View, CA).

2.10 Co-culture assay

C57BL/6 mice were injected with pAAV/HBV1.2 or pAAV/core-null plasmids hydrodynamiclly. The hepatocytes were isolated at 3 days postinjection. The intrahepatic leukocytes were prepared from naïve C57BL/6 mice and cocultured with hepatocytes for 24 hours. Cell-culture supernatants were collected and TNF-α levels were detected by ELISA kit according to manufacturer's instructions (eBioscience, San Diego, CA).

2.11 In vivo treatment of mice with the anti-PD-1 mAb

An anti-mouse PD-1 Ab was purified from hybridoma (clone G4) culture supernatant (kindly provided by Dr. Lieping Chen, Yale University). C57BL/6 mice were intraperitoneally (i.p.) treated with an anti-PD-1 blocking Ab or isotype control Ab. Ab administration was initiated every 2 days on day 6 before injection with the HBV

core-truncated (HBV-175) mutant construct. Thereafter, Abs (200 μg) were repeatedly administered every 3~4 days for a period of 8 weeks.

2.12 Purification of monoclonal antibody from hybridoma

Cell line G4 which secreted monoclonal antibody against mouse PD-1 were cultured in RPMI1640 medium containing 10% FBS and antibiotics. The culture supernatants were collected for further purification. Protein G sepharose column was washed with 100mM Tris-HCL pH 8.0 buffer. The culture supernatant were passed through protein G column. The column was washed and eluted with 50 mM glycine(pH3.0). The antibody concentration was determined for further applications.

Chapter III
Results

3.1 Inhibitory receptor PD-1 in chronic viral infection

Hepatitis B virus (HBV) causes acute and chronic inflammatory liver diseases and subsequent hepatic cirrhosis and hepatocellular carcinoma (HCC). During chronic HBV infection, a dynamic balance between viral replication and the host immune response is pivotal to the pathogenesis of liver disease. It is widely accepted that adaptive immune responses, particularly cellular immune responses, mediate the clearance of HBV (Chisari and Ferrari, 1995; Jung and Pape, 2002) Unfortunately, HBV-specific T-cell function is impaired in patients with chronic HBV infection characterized by low levels of antiviral cytokines, impaired cytotoxic T lymphocyte activity, and persistent viremia (Iwai et al., 2003). However, the mechanism underlying this T-cell malfunction in chronic HBV infection is not completely understood.

The immunologic receptor, programmed death (PD)-1, a 55-kDa transmembrane protein containing an immunologic receptor tyrosine-based inhibitory motif, was originally isolated from a T-cell line exhibiting a high sensitivity to apoptosis (Ishida et al., 1992). The PD-1/PD-L1 pathway is well documented to play a negative role in regulating activation and proliferation of T-cells and production of cytokines (Chen, 2004; Nurieva et al., 2006). There is evidence that the PD-1 pathway plays an important role in inhibiting the function of virus-specific $CD8^+$ T-cells in chronic viral infection involving human immunodeficiency virus (HIV) (Day et al., 2006), hepatitis C virus

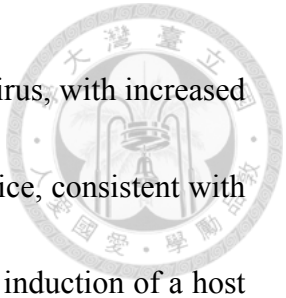
(HCV) (Golden-Mason et al., 2007), and HBV (Boni et al., 2007). Although reports are available on changes in expression levels of PD-1 and T-cell responses in patients with HBV infection, the pattern of change of PD-1 expression in the natural course of chronic HBV infection has not yet been presented.

3.2 Increased PD-1-expressing $CD8^+$ and $CD4^+$ T-cells in liver-infiltrating lymphocytes from mice with HBV persistence

Successful eradication of HBV infection requires elimination of virus-infected hepatocytes and inhibition of virus replication by the host immune system. Although the chronicity of HBV infection is the result of impaired HBV-specific immune responses that cannot efficiently eliminate or cure infected hepatocytes, many issues remain unsettled. We established a mouse model of HBV persistence in immunocompetent mice by a hydrodynamic injection of replication-competent HBV DNA. This approach generated HBV persistence in young C57BL/6 mice, but not in BALB/c mice (Huang et al., 2006; Lin et al., 2010). C57BL/6 and BALB/c mice were hydrodynamically injected with the pAAV/HBV1.2 plasmid. At the indicated time points, intrahepatic lymphocytes from mice that were HBsAg-positive (carrier) or HBsAg-negative (cleared) were isolated, and PD-1 expressions by $CD8^+$ and $CD4^+$ T-cells were analyzed by flow cytometry. The results in Figure 1 demonstrate that there were increased proportions of

PD1-expressing $CD8^+$ and $CD4^+$ T-cells in livers of C57BL/6 mice, but not in BALB/c mice. Moreover, in C57BL/6 mice, both PD-1-expressing $CD8^+$ and $CD4^+$ intrahepatic T-cells significantly decreased in mice which had cleared the injected pAAV/HBV1.2 plasmid (cleared mice), compared to mice with HBV persistence (carrier mice). These results indicate a significant increase in PD-1 expression in both intrahepatic $CD8^+$ and $CD4^+$ T-cells of mice with HBV persistence. In addition, we demonstrated that there were increased PD1-expressing $CD8^+$ T-cells in liver-infiltrating lymphocytes in C57BL/6 mice, which were more susceptible to HBV persistence compared to BALB/c mice; and there were also increased PD1-expressing $CD8^+$ and $CD4^+$ T-cells in HBV carrier mice compared to control mice or mice which had recovered from an HBV infection (Figure 1). This is consistent with the results of recent studies of HBV-infected patients, which demonstrated that PD-1 expression can impair virus-specific CD8 T-cell responses during chronic HBV infection (Hsu et al., 2010; Xie et al., 2009; Yang et al., 2010; Zhang et al., 2009; Zhang et al., 2008).

Furthermore, to further define the role of the HBV core in HBV persistence in this mice animal model, C57BL/6 mice were injected with wild-type (WT) pAAV/HBV1.2 or HBV core mutant DNA, including HBV-175 or HBV-38 (Lin et al., 2010), and HBV persistence and PD-1 expression in liver-infiltrating lymphocytes were analyzed by flow cytometry. Both the HBV-175 and HBV-38 core-mutant viral constructs caused



significant loss of the ability to raise the host immunity to clear the virus, with increased PD-1-expressing T-cell infiltration, leading to HBV persistence in mice, consistent with the observation that the HBV core protein plays an important role in induction of a host immune response to HBV (Figure 2)(Lin et al., 2010)

3.3 Liver-infiltrating $CD8^+$ lymphocytes in carrier mice displayed the $PD\text{-}1^{hi}CD127^{low}$-exhausted phenotype

Recent studies in animal models of viral infection indicated that the interaction between PD-1 on lymphocytes and its ligands plays a critical role in T-cell exhaustion by inducing T-cell inactivation. HBV-specific PD-1-positive CD8 cells of patients with chronic HBV infection also displayed lower levels of the interleukin (IL)-7 receptor, CD127, which was previously described in association with the exhausted phenotype (Boettler et al., 2006; Boni et al., 2007). To define the exhausted phenotype in the PD-1-expressing intrahepatic T-cells in mice with HBV persistence, liver-infiltrating $CD8^+$ lymphocytes from mice hydrodynamically injected with the HBV construct were evaluated for the expressions of both PD-1 and CD127 by $CD8^+$ T-cells by flow cytometry (Figure 3). The results in Figure 3 demonstrate that PD-1 was more highly expressed by intrahepatic CD8 cells in mice with HBsAg persistence. Also, among intrahepatic CD8 populations, CD127 expression was significantly lower in carrier mice

compared to mice which had cleared HBV. Our results indicate that liver-infiltrating $CD8^+$ lymphocytes in carrier mice displayed the PD-$1^{hi}CD127^{low}$-exhausted phenotype.

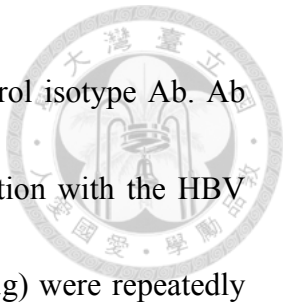
3.4 Liver-infiltrating PD-1^+CD4^+ T-cells in mice with HBV persistence exhibit the phenotype of regulatory T-cells (Tregs)

Our results indicated that there were significant increases in PD-1-expressing $CD8^+$ and $CD4^+$ T-cells in liver-infiltrating lymphocytes from mice with HBV persistence. We further analyzed the immune phenotype of PD-1-expressing intrahepatic $CD4^+$ T-cells. Intrahepatic lymphocytes from C57BL/6 mice that were HBsAg-positive 4 weeks after being hydrodynamically injected with WT pAAV/HBV1.2 were isolated, and the immune phenotype was analyzed. The results in Figure 4 demonstrate that there were significantly increased $CD4^+$ $Foxp3^+$ populations in mice with HBV persistence. Moreover, the expression of CTLA-4 was also upregulated in PD-1^+ $CD4^+$ T-cells, indicating that these PD-1^+ T-cells displayed the Treg phenotype. Taken together, there were increases in PD1-expressing $CD8^+$ T-cells in the liver, and increased PD-1 expressing $CD4^+FoxP3^+$ T-cells and CTLA4-expressing $CD4^+$ T-cells in the liver of HBV carrier mice. The results indicate that PD-1-expressing $CD4^+$ T-cells in liver-infiltrating lymphocytes from mice with HBV persistence were Tregs.

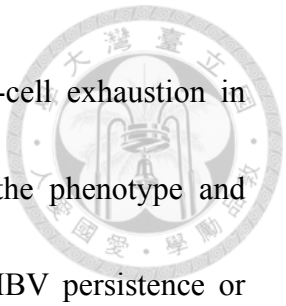
We then asked whether the PD-1/PD-L1 interaction of intrahepatic T-cells was associated with the impaired immune response, resulting in a defective T-cell response to HBV in mice with HBV persistence after an infection. The HBV core-specific IFN-γ T-cell response in mice hydrodynamically injected with WT pAAV/HBV1.2 (HBV-wt), HBV-175, or HBV-38 in the presence and absence of anti-PD-1 mAb treatment were analyzed by an enzyme-linked immunosorbent spot (ELISPOT) assay. Results in Figure 5 demonstrate that the frequency of HBcAg-specific IFN-γ-secreting cells was significantly reduced in mice injected with HBV core mutant constructs; however, impairment of the HBcAg-specific IFN-γ response was restored in mice treated with the anti-PD-1 mAb. Taken together, our results indicate that the impaired T-cell response to the HBV core in the IFN-γ ELISPOT assay could be restored by *in vivo* treatment with an anti-PD-1 mAb, indicating that the level of PD-1 expression on intrahepatic T cells is correlated with the anti-viral T-cell response *in vivo*.

3.5 Blockade of the PD-1 pathway by an anti-PD-1 mAb reduced the HBV persistence rate in this mouse animal model

To further define the role of T-cell exhaustion in the pathogenesis of persistent HBV infection in this mice animal model, and determine the effect of PD-1/PD-L1 blockade on restoring immune dysfunction and clearance of HBV, C57BL/6 mice were



intraperitoneally treated with an anti-PD-1 blocking mAb or a control isotype Ab. Ab administration was initiated every 2 days on day 6 before an injection with the HBV core-truncated (HBV-175) mutant construct. Thereafter, Abs (200 μg) were repeatedly administered every 3~4 days for a period of 8 weeks. The HBsAg level in mice serum was determined by an ELISA. The results in Figure 6 demonstrated that blocking the interaction of PD-1/PD-L1 by the anti-PD-1 mAb significantly reduced the frequency of HBV persistence in mice injected with the core-null HBV viral construct, resulting in clearance of HBV in vivo. The mice treated with anti-PD-1 mAb with higher viral clearance rate and also showed higher ALT level compared to control mice. In mice treated with anti-PD-1 mAb, the PD-1 expression level in liver infiltrating lymphocytes after hydrodynamic infection of HBV was similar to mice treated with control Ig. In contrast, among intrahepatic CD8 populations, CD127 expression was significantly higher in mice treated anti-PD-1 mAb compared to mice treated with control Ig (Figure 7), indicating PD-1 blockage reversed PD-$1^{hi}$$CD127^{low}$-exhausted phenotype in intrahepatic $CD8^{+}$ T cells in mice treated with anti-PD-1 mAb. Taken together, these results indicate that PD-1 blockage reverses immune dysfunction as well as the PD-$1^{hi}$$CD127^{low}$-exhausted phenotype and viral persistence to HBV infection in this mouse animal model, suggesting that the anti-PD-1 mAb might be a good therapeutic candidate for chronic HBV infection.



These results are thus undertaken to further define the role of T-cell exhaustion in chronic HBV infection in a mice animal model, by comparing the phenotype and function of intrahepatic infiltrating T lymphocytes in mice with HBV persistence or HBV clearance, and the effect of PD-1/PD-L1 blockade in restoring immune dysfunction and clearance of HBV. Furthermore, PD-1/PD-L1 blockade partially restored the function of intrahepatic T-cells, leading to viral clearance. It is the first report to demonstrate PD-1/PD-L1 blockade could reverses immune dysfunction and HBV viral persistence *in vivo*. This observation opens new potential perspectives for the development of novel immunotherapies for chronic hepatitis B.

3.6 Induction of host innate immune response by HBV

It has well established that activation of cellular immune response is required for mediating HBV clearance from the liver. In our mouse model of HBV persistence, we demonstrated that the chronicity of HBV infection is associated with PD-1 expression on intrahepatic lymphocytes (Tzeng et al., 2012). It is believed that an efficient control of HBV infection requires the coordinated actions of both innate and adaptive immune responses (Bertoletti and Ferrari, 2012; Durantel and Zoulim, 2009; Yang et al., 2010). Innate immunity induces an antiviral state in infected cells by producing type I interferons (IFN), and supports the efficient maturation and site recruitment of adaptive

immunity through the production of pro-inflammatory cytokines, in particular, tumor necrosis factor-α (TNF-α) (Bertoletti and Ferrari, 2013). However, in chronic HBV infection, impaired HBV-specific immune responses failed to eliminate infected hepatocytes, resulting in the persistence of HBV.

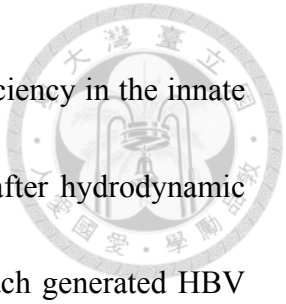
Experimental viral infection in both chimpanzees and woodchucks found only limited or even non-activation of innate immunity being demonstrated in acute HBV infection (Dunn et al., 2009; Guo et al., 2009). Nevertheless, a transient though slight activation of IFN-α genes was detected in human hepatocytes infected by HBV in chimeric mice, in support of the innate immunity to sense and react to HBV (Lutgehetmann et al., 2011). However, the mechanisms responsible for sensing HBV within the infected cells have not been elucidated yet, and which molecular components of the HBV actually recognized by the pattern recognition receptors (PRR) triggering the antiviral response is still undefined. In addition, a number of recent studies have been investigated in suggesting the involvement of NK cells in chronic HBV infection and they could play a role in liver damage during reactivation (Dunn et al., 2007; Oliviero et al., 2009; Peppa et al., 2010). The role of innate immunity in viral clearance during HBV infection is still not clear.

TNF-α has long been considered as a key cytokine in HBV eradication (Larrubia et al., 2009). Higher intrahepatic levels of TNF-α have been associated with the increased

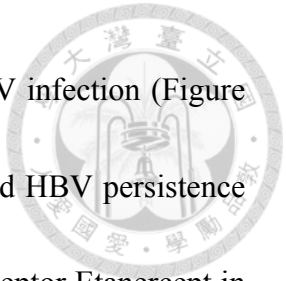
expression of HLA class I molecules and an enhanced $CD8^+$ T cell response to HBV, which leads to the more effective destruction of HBV-infected hepatocytes (Hussain et al., 1994). In chronic HBV infection, $CD8^+$ T cells lack the ability to secrete enough TNF-α to kill HBV-infected hepatocytes, the so-called "exhausted phenotype." This is a functional HBV-specific $CD8^+$ T cell impairment that is detectable at the peak of the disease when the majority of HBV-specific $CD8^+$ T cells are activated but have little ability to proliferate and are functionally exhausted, probably due to upregulation of programmed death (PD)-1 (Fisicaro et al., 2010; Peng et al., 2008b). Studies have demonstrated that genetic polymorphisms leading to lower constitutive or inducible TNF-α expression are related to an increased risk of progression toward chronic HBV infection (Ben-Ari et al., 2003; Hohler et al., 1998). Clinically, an anti-TNF regimen also reportedly increased the number of cases of HBV reactivation (Lan et al., 2011; Perez-Alvarez et al., 2011). However, pro-inflammatory cytokines are often undetectable during the early phases of HBV infection (Wieland et al., 2004).

3.7 The TNF-α rather than IFN-mediated pathway is critical in HBV clearance

Although the chronicity of HBV infection is the result of impaired HBV-specific immune responses that cannot efficiently eliminate or cure infected hepatocytes, however, this likely a result from the failure of immune responses at the first exposure



to HBV. Therefore, we tested a panel of KO mice with specific deficiency in the innate immune sensors or effectors for their capability in clearing HBV after hydrodynamic injection (HDI) of a replication competent HBV DNA. This approach generated HBV persistence in C57BL/6 mice but not in BALB/c mice (Huang et al., 2006; Lin et al., 2010; Tzeng et al., 2012). To identify the immune effectors of innate immunity that eliminate HBV from the liver, we monitored the persistence of HBsAg in a panel of gene knockout mice, including Nod-like receptor family protein 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), myeloid differentiation primary response gene 88 (MYD88), IL-1 receptor (IL-1R), IFN-α/β receptor (IFNAR), and TNF-α. The results (Figure 8) demonstrate that HBV viral clearance is not significantly different from wild type C57BL/6 mice in IFNAR, RIG-I, MDA5, MYD88, NLRP3, ASC, and IL-1R knock-out mice, indicating that these effectors are not required for HBV clearance. In contrast, only TNF-α knockout mice showed a markedly higher HBV-positive rate and prolonged HBV persistence compared to other strains, suggesting that TNF-α is an important effector cytokine that is required to clear HBV from the liver. The results (Figure 8 and Figure A1) demonstrated that the HBV persistence rate and serum HBs Ag levels was similar between IFNR knock-out mice and wild type C56BL/6 mice, indicating that IFN-mediated pathways are not essential for clearing HBV in this animal model. We



further investigated the roles of innate cytokines TNF-α during HBV infection (Figure 9). In contrast, significantly impaired HBV clearance and enhanced HBV persistence was observed when TNF-α was neutralized with the soluble TNF receptor Etanercept in HBV-cleared mouse strain BALB/C, suggesting that TNF-α is required for HBV clearance (Figure 9A). Similarly, the HBV persistence rate and serum HBs Ag levels was significantly enhanced in TNF-α knockout mice compared to wild-type C57BL/6 mice (Figure 9B). Taken together, these results indicate that TNF rather than the IFN-mediated pathway is required for HBV clearance, and TNF blockage enhances HBV persistence in vivo.

3.8 TNF-α deficiency leads to cytotoxic T lymphocyte dysfunction against HBV

We then asked whether TNF-α deficiency is associated with an impaired T cell response to HBV in mice with HBV persistence. Recent studies in viral infection indicate that the interaction between the PD-1 on lymphocytes and its ligands plays a critical role in T-cell exhaustion by inducing T-cell inactivation and displayed lower levels of interleukin (IL)-7 receptor CD127, which had previously been described in association with the exhausted phenotype (Boettler et al., 2006; Boni et al., 2007). The results in Figure 10A demonstrate that PD-1 is more highly expressed by intrahepatic CD8 cells in BALB/c mice hydrodynamically injected with HBV constructs and treated with

Etanercept. Also, among intrahepatic PD-1 -expressing CD8 populations, CD127 expression was significantly reduced in mice treated with Etanercept. Interestingly, there are no such differences noted for the spleen. Our results indicate that there are significantly increased liver-infiltrating PD-$1^{hi}CD127^{low}$-exhausted $CD8^+$ lymphocytes in Etancercept-treated HBV infected mice. Similar results were observed in TNF-α knockout mice (Figure 10B). Both results indicate that liver-infiltrating $CD8^+$ lymphocytes in response to HBV in mice with TNF-α deficiency displayed the PD-$1^{hi}CD127^{low}$-exhausted phenotype.

We then evaluated T cell response to HBV in mice with HBV persistence after an infection. The HBV core-specific IFN-γ T-cell response in mice hydrodynamically injected with HBV DNA in the presence and absence of TNF-α were analyzed by an ELISPOT assay. Results in Figure 10C demonstrate that the frequency of HBcAg-specific IFN-γ-secreting cells was significantly reduced in TNF-α knockout C57BL/6 mice or Etanercept-treated BALB/c mice. Taken together, our results indicate TNF-α is correlated with the anti-HBV T cell response *in vivo*.

3.9 TNF-α blockage-induced elevation of serum HBV viral loads and maintained HBV viral gene expression within the liver

Our results indicate that TNF-α deficiency is associated with the impaired T-cell response to HBV. We then investigated the effects of TNF blockage on HBV viral load and viral replication. The results (Figure 11A) show the control group BALB/c mice were almost cleared of HBV viral loads in the initial five weeks after hydrodynamic transfection of HBV. In contrast, persistently elevated HBV viral loads in serum were observed in BALB/c mice treated with Etanercept (Figure 11A). TNF-α knockout mice showed similar results with persistently elevated HBV viral loads in serum compared to the wild type C57BL/6 mice (Figure 11B). We then analyzed the HBV transcripts in the livers of mice transfected with /HBV by Northern blotting after hydrodynamic injection (Figure 12). The HBV transcripts remained detectable in the liver of TNF-α knockout and Etanercept-treated BALB/c mice up to day 42 post-transfection. In contrast, the HBV transcripts were almost undetectable in wild type C57BL/6 mice on day 42 post-transfection. Similarly, the results from an immunohistochemistry analysis also revealed that the staining for HBcAg and HBsAg remained detectable in the livers of TNF-α knockout and Etanercept-treated BALB/c mice on day 42 post-transfection. However, HBcAg and HBsAg staining was much lower in wild type C57BL/6 mice on day 42 post-transfection, which is correlated with serum viral loads and HBV transcripts in the liver (Figure 12B). These results indicate that a TNF-α deficiency impairs viral clearance and increases HBV viral load and viral replication *in vivo*.

3.10 Lack of TNF-α eliminates HBcAg-induced HBsAg clearance

Previous studies showed that the immune response triggered in mice by HBcAg during exposure to HBV is important in determining HBV clearance (Lin et al., 2010; Tzeng et al., 2012; Yang et al., 2013). We then test whether HBcAg can induce cytokines production *in vivo*. C57BL/6 mice were hydrodynamic injected with purified recombinant HBcAg, the serum level of TNF-α was increased at 4 hours postinjection. In contrast, administration of exudates from anti-HBc Ab absorption could not induce TNF-α production (Figure 13). Interestingly, the induction of TNF-α by HBcAg was abolished after administration of HBcAg capsid assembly- defective mutant HBcY132A (Figure 14). These results indicated that icosahedral structure of HBcAg protein was required for TNF-α induction. To further define the role of TNF-α on the HBV core-induced HBV clearance in this mouse animal model, BALB/c mice were hydrodynamically injected on day -7 with pAAV/core-null plasmids containing a core-deleted HBV construct (Lin et al., 2010). The mice were then treated with purified recombinant HBV core protein in the presence or absence of Etanercept by hydrodynamic injection. Serum levels of HBsAg significantly decreased after pretreatment with recombinant HBV core protein (Figure 15A). However, in mice treated with Etanercept, the effect of HBV core-induced clearance of HBsAg was eliminated. In addition, the introduction of a capsid assembly-defect mutant form of

HBcY132A (Lin et al., 2012) failed to reduce the serum level of HBsAg (Figure 15B). However, co-injection of pAAV/e/c-null HBV plasmids with a plasmid encoding HBcAg efficiently decreased HBsAg levels. In contrast, when administered with Etanercept to neutralize TNF-α, the effect of HBV core-induced HBsAg clearance was abrogated. Similarly, the effect of HBV core-induced HBsAg clearance was also diminished in TNF-α knockout mice having elevated levels of HBsAg after hydrodynamic transfection with HBV (Figure 15C). Taken together, these results indicate that blockage of TNF-α inhibits the effects of HBV core-induced HBV clearance in this mouse animal model.

3.11 Intra-hepatic leukocytes, in contrasting with HBcAg containing hepatocytes, were responsible for HBcAg-induced TNF-α production

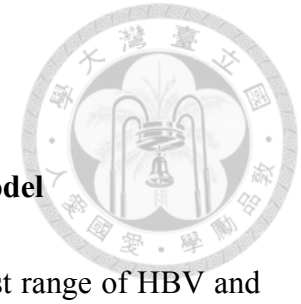
Our results indicate that TNF-α is required for an effective T-cell response to HBV and the immune response triggered in mice by HBcAg during exposure to HBV is critical in HBV clearance. Since various cell types, including innate immune cells and T lymphocytes, secret TNF-α, we then test whether the induction of TNF-α at different time course during HBV exposure influences the host cytotoxic T cell response against this virus. The kinetics of TNF-α in influencing HBsAg persistent rate was performed. BALB/c mice were treated with Entanercept via single-dose injection intravenously at

day -1, day 3 or day 13 (The time point of mice receiving pAAV/HBV1.2 plasmid was defined as day 0). Interestingly, mice receiving administration of Entanercept at day 3 lead to highest HBsAg persistent rate and lowest anti-HBs antibody level. In contrast, mice treated with Entanercept at day 13 developed highest anti-HBs antibody titer and lowest HBsAg persistent rate (Figure 16). These results suggested that TNF-α production in the early phase of HBV infection, most likely from innate immune cells plays critical role in viral clearance. We then investigated the cellular source of TNF-α, which is responsible for HBV core-induced HBV clearance. We used an ex vivo system with isolation of intra-hepatic leukocytes (IHL) from mice receiving pAAV/HBV1.2 or pAAV/core-null plasmids and co-cultured with primary syngeneic hepatocytes. The results (Figure 17) demonstrated that the IHLs, but not the hepatocytes, were the cell source responsible for TNF-α production induced by HBcAg in this mouse animal model. There was no TNF-α production when adding the IHLs isolated from mice receiving pAAV/core-null plasmids or from TNF-α deficient mice in this ex vivo co-culture. Taken together, our results indicate that the production of HBcAg-induced TNF-α by IHLs is required for an effective T cell response to HBV.

In this study, we investigated the role of TNF-α in viral clearance and persistence in a murine model of HBV persistence. The model was used to identify the viral antigen crucial for HBV persistence, and it demonstrated that knocking out HBcAg led to HBV persistence

in mice, indicating that HBcAg is critical in determining HBV persistence. We demonstrate here that a deficiency of TNF-α reduces viral clearance and increases HBV persistence in this mouse model, indicating that TNF-α is crucial for mounting an effective anti-HBV immune response.

Chapter IV
Discussion



4.1 Immune suppression of intrahepatic T lymphocytes in hydrodynamic-based transfection of HBV persistence mouse model

The major obstacle in the study of HBV infection is the limited host range of HBV and hence the unavailability of appropriate animal models. To meet the requirement of a mouse model resembling natural chronic HBV infection in humans, we establish a mouse model with a hydrodynamic injection of replication-competent HBV DNA. This is the first mouse model of chronic HBV infection in which mice liver cells persistently harbor replicating HBV DNA and express HBV transcripts and proteins (Huang et al., 2006; Lin et al., 2010). This non-transgenic mouse model for HBV persistence provides opportunities to investigate the mechanisms of HBV persistence. The model is used to identify the viral antigen(s) crucial for HBV persistence. Our study indicate that knocking out HBcAg, but not HBeAg or pol, lead to HBV persistence in mice, and the essential region of HBcAg is the carboxyl terminus, specifically the 10 terminal amino acids (HBcAg176~185) (Lin et al., 2010). These results indicate that the immune response triggered in mice by HBcAg during exposure to HBV is important in determining HBV persistence. Tolerance toward the HBV surface antigen in this model is shown to be due to insufficient cellular immunity against the hepatitis B core antigen, as is documented in humans. In this study, we demonstrate that there are increased intrahepatic PD-1-expressing $CD8^+$ and $CD4^+$ $FoxP3^+$ T-cells in mice with HBV

persistence. Intrahepatic-infiltrating $CD8^+$ T-cells express higher levels of PD-1 and lower levels of CD127 in mice with HBV persistence. Furthermore, PD-1/PD-L1 blockade partially restore the function of intrahepatic T-cells, leading to viral clearance. Although there are studies with similar observations from HBV transgenic mice (Maier et al., 2007) and clinical subjects (Fisicaro et al., 2010; Xie et al., 2009; Zhang et al., 2008), it is the first report to demonstrate PD-1/PD-L1 blockade could reverses immune dysfunction and HBV viral persistence *in vivo*. Moreover, even though reports are available on changes in expression levels of PD-1 and T-cell responses in patients with HBV infection, the pattern of change of PD-1 expression in the course of HBV infection *in vivo* has not yet been presented. The results from our study open new potential perspectives for the development of novel immunotherapies for chronic hepatitis B.

Recent studies using animal models of viral infection in both mice and macaques indicate that the interaction between the inhibitory PD-1 on lymphocytes and its ligand (PD-L1) plays a critical role in T-cell exhaustion by inducing T-cell inactivation, and high PD-1 expression levels by peripheral T-lymphocytes and the possibility of improving T-cell function by blocking PD-1-mediated signaling confirm the importance of this inhibitory pathway in inducing T-cell exhaustion (Barber et al., 2006; Maier et al., 2007; Velu et al., 2009). Furthermore, inhibition of the HBV-specific CTL response and IFN-γ secretion was found to be exerted by PD-1/PD-L1 interactions in the liver

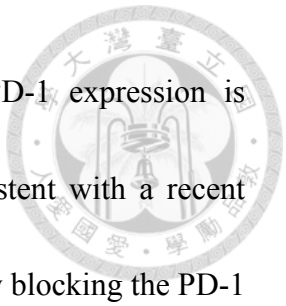
(Maier et al., 2007; Peng et al., 2008b; Xie et al., 2009; Zhang et al., 2009; Zhang et al., 2008). Moreover, *in vivo* manipulation of costimulatory pathways to restore the antiviral function of exhausted T-cells was successfully applies in mice persistently infected with a lymphocytic choriomeningitis virus (LCMV) to improve therapeutic vaccinations (Ha et al., 2008). Also, in various human chronic infections, including hepatitis B, high PD-1 levels are expressed by virus-specific T-cells, and improved T-cell function was obtained *in vitro* by inhibiting the PD-1/PD-L1 interaction.

Our results also demonstrate there are increased PD-1-expressing $CD8^+$ and $CD4^+$ T-cells in mice infected with core mutants with viral persistence, indicating the HBV core protein, in particular the terminal 10 amino acids, is critical for inducing immunity to HBV. We also find that there are increased PD1-expressing $CD8^+$ T-cells in the liver and increased PD-1-expressing $CD4^+FoxP3^+$ Treg cells and CTLA4-expressing $CD4^+$ T-cells in the liver of HBV persistently infected carrier mice (Figure 4), suggesting an active intrahepatic immune suppression process of the adaptive immune system during chronic HBV infection. In persistent HBV infection, it was found that the adaptive immune system, instead of engaging in viral clearance, exerts active immune suppression which inhibits HBV clearance and promotes the development of immune tolerance (Boni et al., 2007; Maier et al., 2007). In this respect, significantly increased $CD4^+CD25^+$ Treg cells in both the circulation and liver-infiltrating lymphocytes were

found in chronic hepatitis B patients and decreased HBcAg-specific $CD4^+CD25^+$ Treg cells were correlated with the occurrence of acute exacerbations during chronic hepatitis B infections (Feng et al., 2007). In vitro, $CD4^+CD25^+$ Treg cells suppressed proliferation of peripheral blood mononuclear cells (PBMCs) in response to HBV antigens stimulation, and depletion of Treg cells led to increased IFN-γ production by PBMCs (Stoop et al., 2007a; Stoop et al., 2007b; Xu et al., 2006). Furthermore, inhibition of the HBV-specific CTL response and IFN-γ secretion was found to be exerted by PD-1/PD-L1 interactions in the liver (Maier et al., 2007; Peng et al., 2008b; Xie et al., 2009; Zhang et al., 2009; Zhang et al., 2008).

4.2 Antiviral response of intrahepatic T-cell can be restored by blocking PD-1 pathway *in vivo*

In a previous study, we isolated liver-infiltrating lymphocytes from liver tissues of chronic hepatitis B patients and analyzed their immune phenotype. We found a significantly increased $CD8^+$ T-cell population with a $CD45RO^+$ $CD69^+$-activated memory phenotype with increased PD-1 and decreased CD28 (Hsu et al., 2010). These data are consistent with the findings in this mouse model and suggest a tolerant immune response underlying chronic HBV infection in both humans and mice. Furthermore, the impaired T-cell response to the HBV core in the IFN-γ ELISPOT assay was restored by



treatment with the anti-PD-1 mAb (Figure 5), indicating that PD-1 expression is correlated with the *in vivo* antiviral T-cell response. This is consistent with a recent report that antiviral intrahepatic T-cell responses could be restored by blocking the PD-1 pathway *ex vivo* in patients with chronic hepatitis B infection (Fisicaro et al., 2010). We further demonstrated that PD-1 blockage reversed immune dysfunction and viral persistence to HBV infection in this mouse animal model. This is the first report to demonstrate that PD-1 blockage can reverse immune dysfunction and HBV persistence *in vivo*, indicating that anti-PD-1 might be a good therapeutic candidate for chronic HBV infection.

Studies in animal models of viral infections show that persistent exposure to high antigen concentrations can affect the antiviral T-cell function by causing different degrees of functional impairment, up to physical T-cell deletion, as a function of the quantity of antigens to which T-cells are exposed (Wherry et al., 2007). T-cell exhaustion by this mechanism may play an important role in the pathogenesis of HBV-specific T-cell hyporesponsiveness with chronic HBV infection, because high antigen concentrations are consistently present at all stages of the infection. The importance of T-cell exhaustion in chronic HBV infection was confirmed by the high expression of PD-1 by circulating HBV-specific CD8 cells and by the possibility of partially improving the peripheral blood T-cell function by blocking the interaction of

PD-1/PD-L1 with anti-PD-L1 Abs (Boni et al., 2007). Moreover, additional support for exhaustion is provided by the inverse correlation between the function of HBV-specific T-cells and viremia levels, since T-cells are more profoundly inhibited in the presence of high viremia (Boni et al., 2007; Maini et al., 2000). Because T-cells are an essential component of antiviral protection, restoration of efficient T-cell function may represent a strategy to cure infections, and manipulation of costimulatory pathways involved in T-cell activation may be a rational approach to achieve this goal. The results obtained in this study that PD-1 blockage reverses immune dysfunction as well as the PD-$1^{hi}$$CD127^{low}$-exhausted phenotype, and viral persistence to HBV infection in this mouse animal model further support this notion.

In conclusion, in this study, we demonstrated that blockade of the PD-1/PD-L1 interaction increased IFN-γ production in response to the HBV core by intrahepatic lymphocytes. Furthermore, blocking the interaction of PD-1 with its ligand, PD-L1, by an anti-PD-1 mAb reversed the viral persistence to clearance of the core-null HBV viral construct in this mouse animal model. Our results indicate that PD-1 blockage reverses immune dysfunction and viral persistence to HBV infection in this mouse animal model, suggesting that anti-PD-1 might be a good therapeutic candidate for chronic HBV infection.

4.3 TNF-α contributes to induce an effective T cell response and lead to HBV clearance

In this study, we demonstrate that a TNF-α blockade using a soluble TNF-α receptor, Etanercept, or mice with a TNF-α gene deficiency all suffer delayed viral clearance and enhanced HBV persistence in this mouse model. Previous studies revealed that several innate inflammatory cytokines had potential modulating effects on HBV gene expression, including IFN-α, IL-1α, IL-6 (Hosel et al., 2009), and TNF-α (Brunda and Rosenbaum, 1984; Gilles et al., 1992; Neta et al., 1988). However, due to experimental limitations, only limited or even non-activation of innate immunity could be demonstrated in acute HBV infection. The role of innate immunity on HBV clearance is still uncertain. To address the role of innate immune responses in HBV clearance, we examined a panel of knock-out mice by a single hydrodynanmic injection of HBV DNA. In this study, after examining the gene-specific knockout mice panels, we found the three major innate pathways (RIG-I, NOD, inflammasome) to be dispensable for HBV clearance. However, a deficiency of TNF-α reduces viral clearance and increases HBV persistence in this mouse model, indicating that the innate cytokine TNF-α is crucial in HBV clearance. The role of the immune effectors required in HBV viral clearance has been studied previously in a panel of immunodeficient mouse strains (Yang et al., 2010). However, only $CD8^+$ T cells were determined to be the key cellular effectors mediating

HBV clearance from the liver, and the roles of TNF-α and other innate effectors in HBV viral clearance were not addressed (Yang et al., 2010). To clarify the effects of innate effectors on HBV clearance, we used a panel of innate effector knockout mice to study their effects on HBV persistence in this mouse model.

Our results indicate that HBV triggers innate immunity via a TNF-α-dependent process to induce an effective T cell response to HBV. The results are consistent with findings in previous study by Yang et. al. concluding that TNF-α is required to eliminate both the HBV DNA and HBcAg from the liver (Yang et al., 2010). Although IFN-α/ß receptor deficiency mice seemed to be impaired in their ability to eliminate the transcriptional template from the liver in that study, their ability to clear HBcAg remained intact (Yang et al., 2010), suggesting that the IFN pathway was not sufficient to clear the HBV and therefore might play a supporting role in HBV clearance. This is consistent with the results of other studies in humans and animal models that type I IFNs are often undetectable during the early phases of HBV infection, and when present their production is decreased (Dunn et al., 2009; Lan et al., 2011). Accumulating evidence suggests that HBV infection induces host immunotolerance (Huang et al., 2006; Xu et al., 2013a). Persistent HBV infection sustains the suppression of antiviral immunity, and high HBV titers or particle loads can inhibit innate immune response activation, particularly innate PRRs and their downstream signals in hepatocytes

(Bertoletti and Ferrari, 2012; Wang and Ryu, 2010; Yu et al., 2010). Nevertheless, in support of the innate immunity to sense and react to HBV, in this study, our results indicate that HBV triggers innate immunity via a TNF-dependent process to induce an effective T cell response to HBV.

As an innate cytokine, the induction of TNF-α not only triggers an inflammatory response but also activates adaptive immunity against HBV and may regulate the balance of virus replication and clearance within the liver. In this study, blockade of TNF-α via single dose administration of Entanercept at the early phase of HBV infection prolonged HBV persistence effectively (Figure 16). Mice with TNF-α deficiency demonstrated an increase in exhausted-phenotype of $CD8^+$ T cells and impaired T cell response to HBV (Figure 10). In addition, TNF-α blockade significantly increased the serum HBV DNA, the expression of HBV core, and HBV viral replication within the liver, indicating that innate cytokine TNF-α is crucial for mounting an effective anti-HBV immune response.

Our results also demonstrate that the HBV core is critical for inducing TNF-α to clear HBV and for TNF blockage to eliminate HBV core-induced viral clearance effects in mice. It implies that the HBV core induces TNF-α through an innate immune sensor to trigger a host anti-HBV immune response that leads to viral clearance. However, the mechanisms responsible for sensing HBV within the infected cells, and which

molecular components of the HBV DNA, RNA or viral proteins are actually recognized by the PRR triggering the antiviral response is still undefined. In this study, our results demonstrate that the HBV core is critical for inducing TNF-α and enhancing the clearance of HBV. In addition, TNF-α blockage abolished the HBV core-induced viral clearance effects in mice (Fig. 15), suggesting that host innate immunity senses the HBV core through the innate immune sensor and induces TNF-α.

In addition, the introduction of a capsid assembly-defect mutant form of HBcY132A failed to trigger the HBV core-induced viral clearance effects (Fig. 15B), suggesting the assembled viral capsid is critical for sensing HBV within the infected cells through the innate immune sensor.

4.4 Intact structure of HBcAg is critical for triggering innate immune response

Previously, our results demonstrated that HBcAg was the major determinant of viral clearance in this mouse model (Lin et al., 2010). Both of the C-terminal truncated mutant of HBcAg and the assembly-defective mutant HBcY132A failed to induce HBcAg-specific IFN-γ production and might result in viral persistence (Lin et al., 2010; Lin et al., 2012). Since the C-terminal truncated mutant of HBcAg, such as HBc166 and HBc175, are able to form HBV capsid, the intact HBcAg that contains icosahedral capsid structure and nucleic acid-binding C terminus are sufficient for triggering

immune response against HBV. Lau et al. demonstrated that transfer of HBV core-specific T cells is associated with recovery of chronic HBV infection and anti-HBs seroconversion (Lau et al., 2002). Moreover, HBcAg-specific IFN-γ production is elevated in T lymphocytes, which are isolated from self-limited hepatitis B patients (Szkaradkiewicz et al., 2003). This is consistent with our results indicating that HBcAg serves as the critical immunogenic viral antigen upon HBV infection. Although HBV has long been considered as a stealth virus since the genes expression of innate-related cytokines are undetectable in HBV-infected chimpanzees (Wieland et al., 2004), accumulating evidences support that HBV can activate innate antiviral response (Fisicaro et al., 2009; Guy et al., 2008; Lucifora et al., 2010). In our studies, administration of purified recombinant HBc capsid, but not free form of HBcAg, induces proinflammatory cytokines such as TNF-α and IL-6 production in mice sera (Figure 13 and 14). Moreover, blockade of TNF-α at the early phase during HBV infection significantly prolongs viral persistence and dampens adaptive immune response, raising the possibility that HBc antigen may act as a molecular pattern that is recognized by innate sensor(s) (Figure 16). Apparently, the icosahedral structure of HBcAg plays important role in inducing immune response, we therefore hypothesize that viral icosahedrons can be sensed by a family of innate sensors to trigger antiviral response which in turn activates T cell immunity, resulting in viral clearance. Most viral

capsid consists of icosahedral structure, so it has the potency to serves as the molecular pattern to be recognized by innate receptor(s). Indeed, recently published reporters have shown that the viral capsid of HIV-1 and adenovirus function as pathogen pattern to be sensed by innate immune sensors (Chintakuntlawar et al., 2010; Pertel et al., 2011). Our results along with these findings collectively imply a novel ligand requirement for unknown family of innate sensors.

The next intriguing question is: which cell types are responsible for sensing HBV capsid? Although the hepatocytes are the natural host cells for the beginning of life cycle of HBV, parenchymal cells of the liver seems not responsible for induction of innate immune response by HBV. Instead, nonparenchymal cells such as Kupffer cells may recognize HBV to mount innate immune response, although they are not infected (Hosel et al., 2009). However, Kupffer cells may contribute to immune tolerance toward HBV by secretion of IL-10, which subsequently promotes type I regulatory T cells differentiation and suppresses adaptive immune response (Xu et al., 2013b, c). Our results demonstrate that intrahepatic leukocytes may be the cell populations that are responsible for TNF-α production (Figure 17). The cell types within intrahepatic leukocytes may play the important role in recognizing HBV capsid to mount TNF-α production. Interestingly, intrahepatic myeloid-cell aggregates for T cell population expansion (iMATE) is proposed to be the important structure for activate CTL to

eliminate virus in the liver. The intrahepatic $CD11b^+$ myeloid cells secrete TNF-α during acute viral infection. TNF-α-mediated signallings cause iMATE formation and break the immune tolerance during chronic viral infection (Huang et al., 2013). Thus, the $CD11b^+$ myeloid cells may be the candidate cells for sensing HBV capsid to produce TNF-α.

NK cells are the major populations of leukocytes in liver and this intrahepatic abundance underscores their critical role in dealing with infections of hepatotropic viruses such as HBV. Several reports have uncovered that NK cells are able to exert antiviral activity and immunoregulatory functions during HBV infection. The antiviral role of NK cells in HBV infection can be exerted directly or indirectly through modulation of T cell response. Secretion of IFN-γ by NK cells promotes T cell response against HBV (Waggoner and Kumar, 2012). NK cells can directly involve in lysis of infected hepatocytes through perforin/granzyme mechanism or death receptor-mediated pathway. However, these mechanisms of viral clearance will destroy the infected hepatocytes. Thus, non-cytolytic mechanisms of HBV clearance through IFN-γ producing NK cells are thought to play important role in viral control (Guidotti et al., 1996). The antiviral role of NK cells also has been verified in transgenic and hydrodynamic-based mouse models of HBV infection (Yang et al., 2002). However, several evidences are highlighting the capacity of NK cells to inhibit T cell response to

limit their antiviral potential (Lang et al., 2012; Waggoner et al., 2010). The contribution of NK cells to control HBV is still controversial. NK cells are also potent TNF-α source. NK-derived TNF-α production may mediate liver injury during HBV infection (Mizuhara et al., 1994). TNF-α-induced hepatocytes death has been shown to be modulated by viral infection, which sensitizes hepatocytes to induction of caspase-dependent apoptosis (Wohlleber et al., 2012). TNF-α also cooperate with FasL to induce hepatocytes apoptosis (Schmich et al., 2011). Thus, NK cells may involve in HBV clearance through TNF-α secretion.

4.5 IFN-mediated pathways are not required for HBV clearance *in vivo*

IPS-1 has been shown to mediate RIG-I/MDA5 signaling to activate interferon regulatory factor 3 (IRF3), leading to phosphorylation of IRF3. Phosphorrylated IRF3 is dimerized and translocated to nucleus to induce the transcription of IFN-β. Binding of IFN-β to type I IFN receptor amplifies innate response by triggering the activation of JAK/STAT pathway. The activation of JAK leads to STAT protein phosphorylation, translocation to the nucleus, and induction of a wide variety of interferon-stimulated genes, such as RIG-I, MDA5 etc. Our results demonstrate that HBV core antigen is responsible for triggering type I interferon-mediated pathway and IPS-1 dependent IRF3 activation (Figure A2, A3), suggesting that RLR-dependent signalings are

responsible for triggering IRF3 activation upon HBV infection. However, the signaling pathway triggered by interaction of HBV core protein and MDA5, but not RIG-I, seems not sufficient for viral clearance (Figure A4 and Figure 8). These results are consistent with recently published report, indicating that MDA5, but not RIG-I, interacts with HBV nucleic acids and inhibits viral replication, but the downstream signalings activation (mainly type I IFN induction) are not sufficient for HBV clearance (Lu and Liao, 2013; Yang et al., 2010). Thus, the innate sensors that recognize the HBV capsid and trigger efficient immune response for viral elimination still await further investigation.

4.6 Working hypothesis of HBV-induced immunity in this mouse model

Lack of the cellular entry of HBV simplifies the interactions between virus and host in this hydrodynamic-based mouse model, but provides the opportunity to manipulate the immune responses induced by HBV. A working hypothesis for HBV-induced host immunity is shown in Figure 18. In viral part, the critical factor for viral clearance is icosahedral structure of HBcAg (Lin et al., 2010). In host part, our results demonstrates that TNF-α is required for effective T cell response against HBV, leading to elimination of viral loads, inhibition of viral genes expression and viral clearance. The release of TNF-α from intrahepatic leukocytes is mediated by HBcAg-expressing hepatocytes.

Nevertheless, we cannot rule out the possibility that HBV also induces immune suppression along with the immune activation arm. Indeed, Kupffer cells are the cell type that mediate humoral immune tolerance toward HBV by IL-10 induction (Xu et al., 2013a). Moreover, secretion of IL-10 promotes type I regulatory T-like cells differentiation, resulting in suppression of germinal centers formation in HBV-persistent mice (Xu et al., 2013c). Base on these findings, we hypothesize that expression of viral antigens in hepatocytes shapes the response of intrahepatic immune cells toward HBV, both immune activation and suppression arms.

Anti-TNF-α is effective for the treatment of rheumatoid arthritis, seronagative spondyloarthropathy, and inflammatory bowel disease. However, there are limited reports on its effect on HBV persistence and reactivation in chronic HBV infection during anti-TNF therapy. We demonstrate in this study that TNF-α blockage reduces viral clearance, induces elevated serum HBV viral loads, enhances HBV viral gene expression, and increases HBV persistence in a mouse model. Therefore, treatment with TNF-α blockage agents may reduce the clearance of HBV and enhance HBV replication and viral persistence. In conclusion, TNF-α deficiency significantly increased serum HBV DNA and viral replication within the liver, indicating that TNF-α is crucial for mounting an effective anti-HBV immune response. Thus, our results provide evidence

that therapy with TNF-α blockage agents may impair the T cell response and enhance viral replication during chronic HBV infection.

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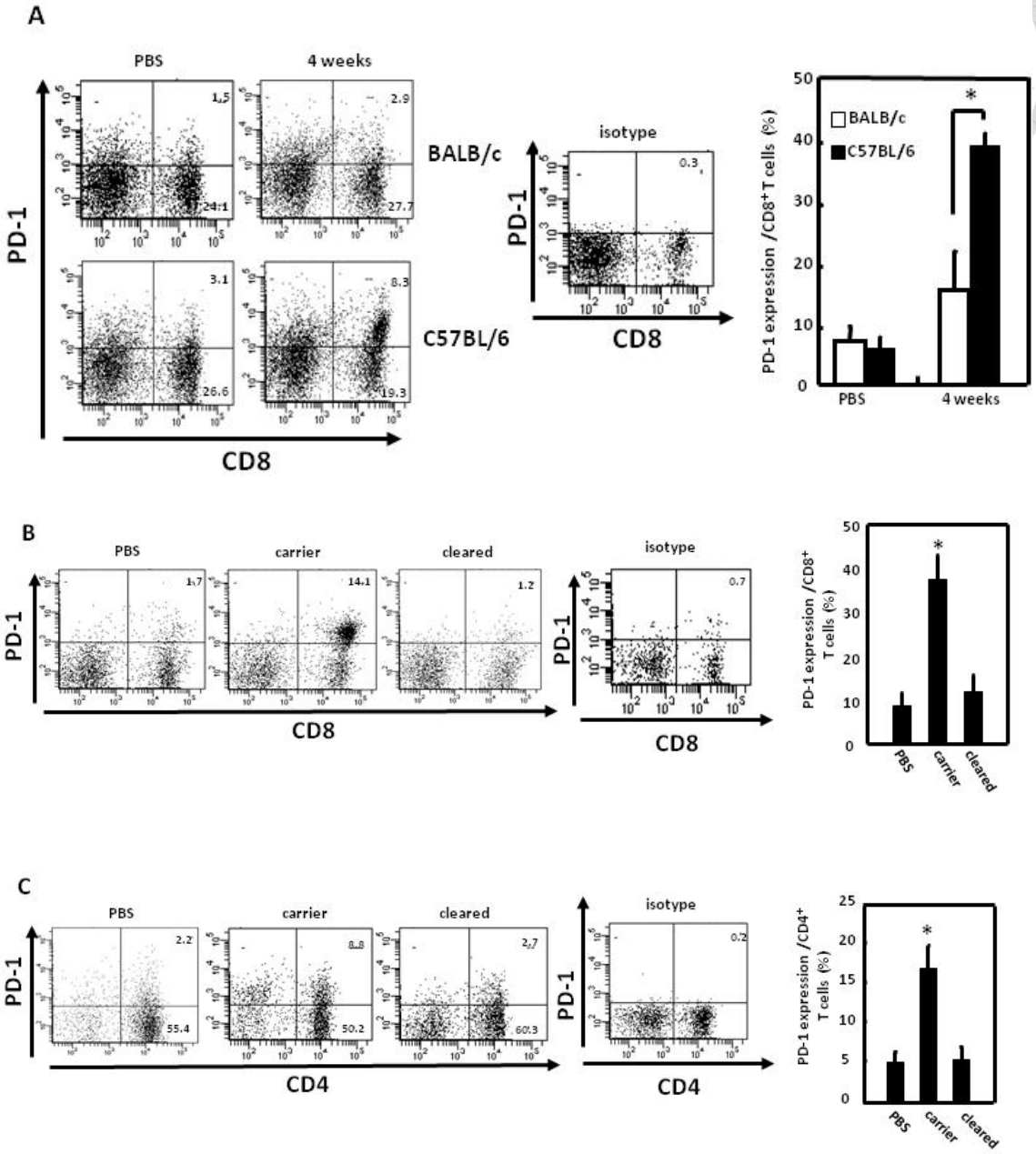
Figures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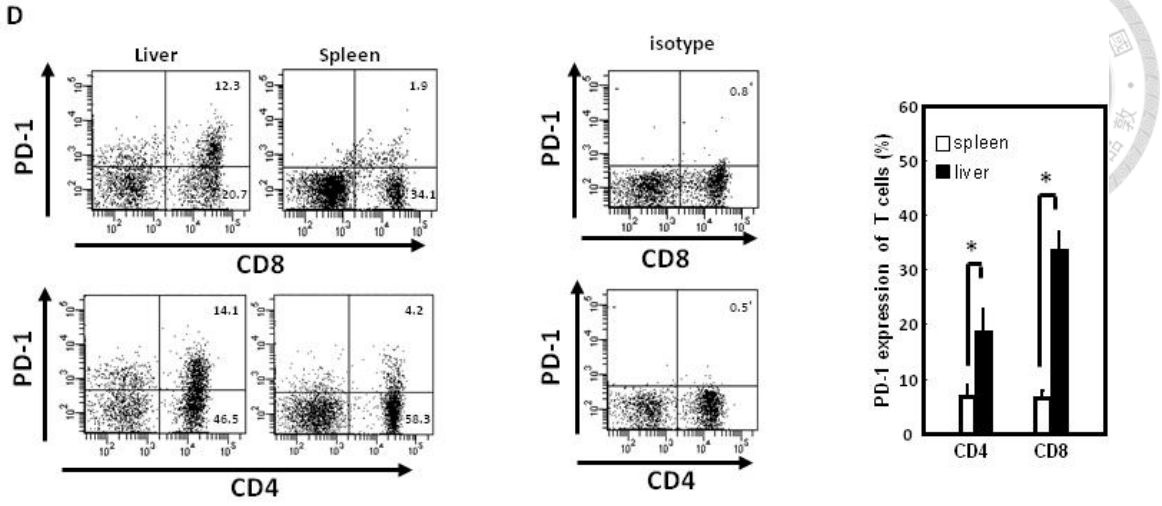


Figure 1. Increased programmed death (PD)-1-expressing $CD8^+$ and $CD4^+$ T-cells in liver-infiltrating lymphocytes from mice with hepatitis B viral (HBV) persistence

A. C57BL/6 or BALB/c mice were hydrodynamically injected with the pAAV/HBV1.2 plasmid. Four weeks post-injection, intrahepatic lymphocytes were isolated and PD-1 expression was analyzed by flow cytometry. B and C. C57BL/6 mice were hydrodynamically injected with pAAV/HBV1.2. Four weeks after hydrodynamic injection, intrahepatic lymphocytes from mice which were HBsAg-positive (carrier) or HBsAg-negative (cleared) were isolated, and the PD-1 expressions by $CD8^+$ (B) and $CD4^+$ (C) T-cells were analyzed by flow cytometry. The data were representative of at least 12 independent experiments. D. C57BL/6 mice were injected with pAAV/HBV1.2 plasmid hydrodynamically. HBsAg-positive carrier were sacrificed at 4 weeks post-injection. Intrahepatic lymphocytes and splenocytes were isolated and the

populations of $CD4^+PD\text{-}1^+$ or $CD8^+PD\text{-}1^+$ were determined by flow cytometry analysis.

* $p<0.05$.

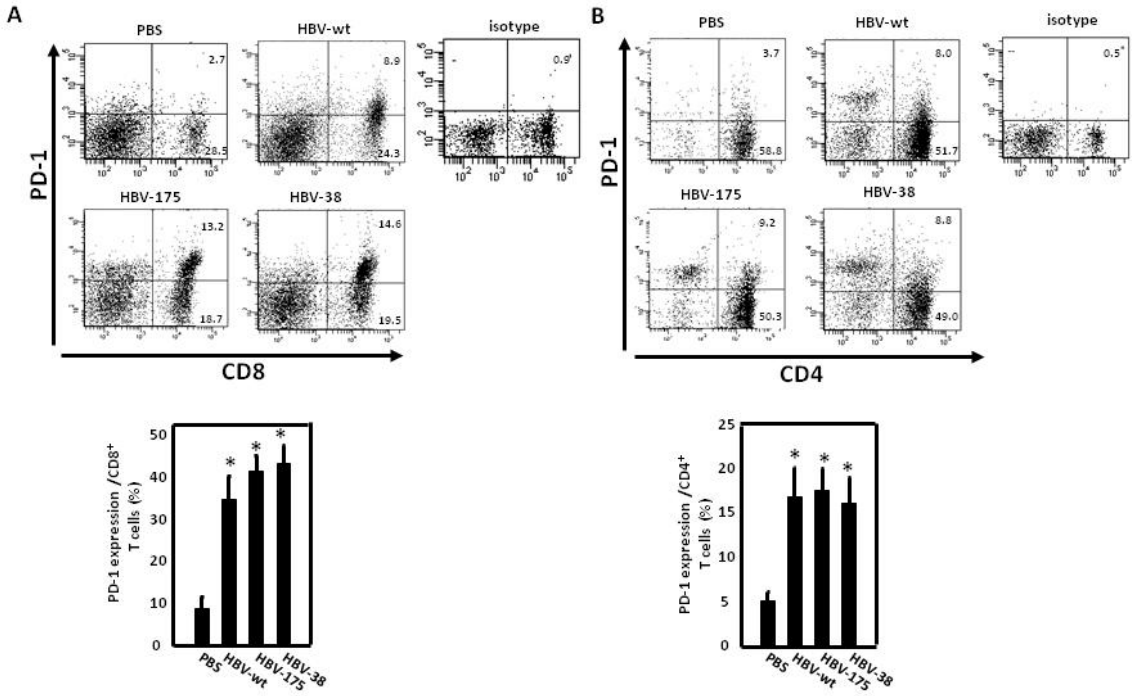


Figure 2. Hepatitis B viral (HBV) core mutants induced upregulation of programmed death (PD)-1 expression in intrahepatic T lymphocytes

C57BL/6 mice were hydrodynamically injected with WT pAAV/HBV1.2 or HBV core mutant DNA, including HBV-175 or HBV-38 constructs. Four weeks after the injection, intrahepatic lymphocytes were isolated, and the PD-1 expressions by $CD8^+$ (A) and $CD4^+$ (B) T-cells were analyzed by flow cytometry. * $p<0.05$ **.** The data were representative of at least 6 independent experiments.

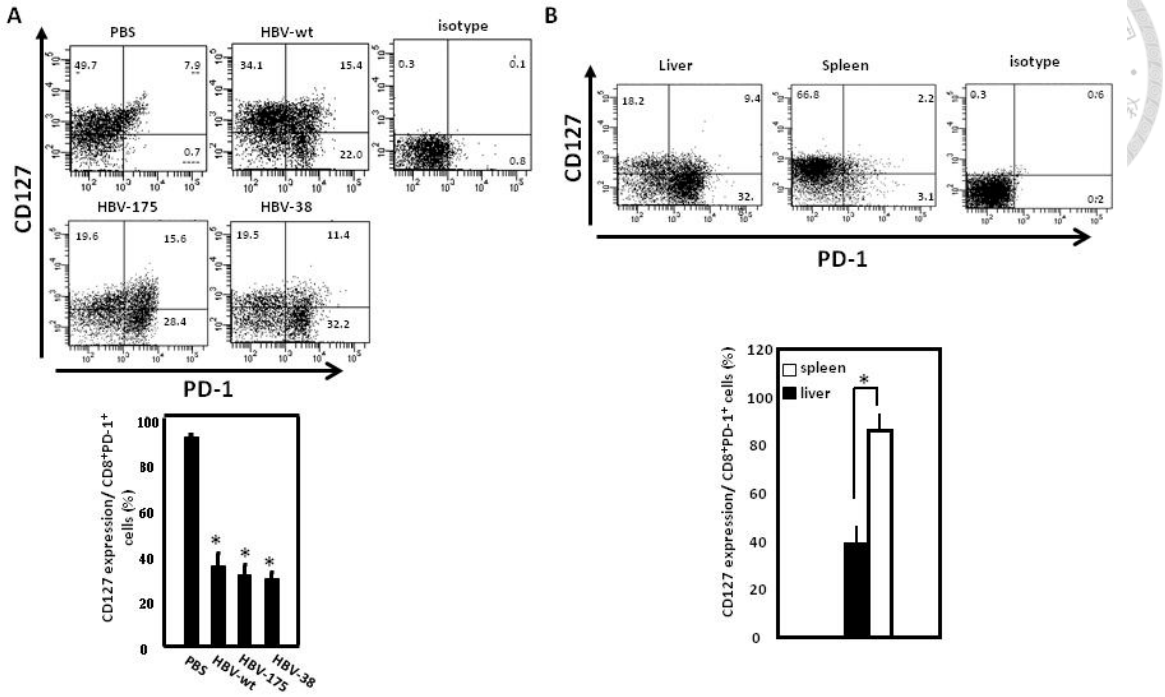


Figure 3. Liver-infiltrating $CD8^+$ lymphocytes in carrier mice displayed the $PD\text{-}1^{hi}CD127^{low}$-exhausted phenotype

(A) C57BL/6 mice were hydrodynamically injected with wild type hepatitis B virus (HBV-wt) and HBV core mutants, including the HBV-175 and HBV-38 constructs. Intrahepatic lymphocytes were isolated at 4 weeks, and PD-1 and CD127 expressions by $CD8^+$ T cells were analyzed by flow cytometry. (B) At 4 weeks post-injection, liver infiltrating lymphocytes and splenocytes were isolated from C57BL/6 mice. The positive staining of PD-1 and CD127 on $CD8^+$ T cells were determined by flow cytometry analysis. * $p<0.05$.

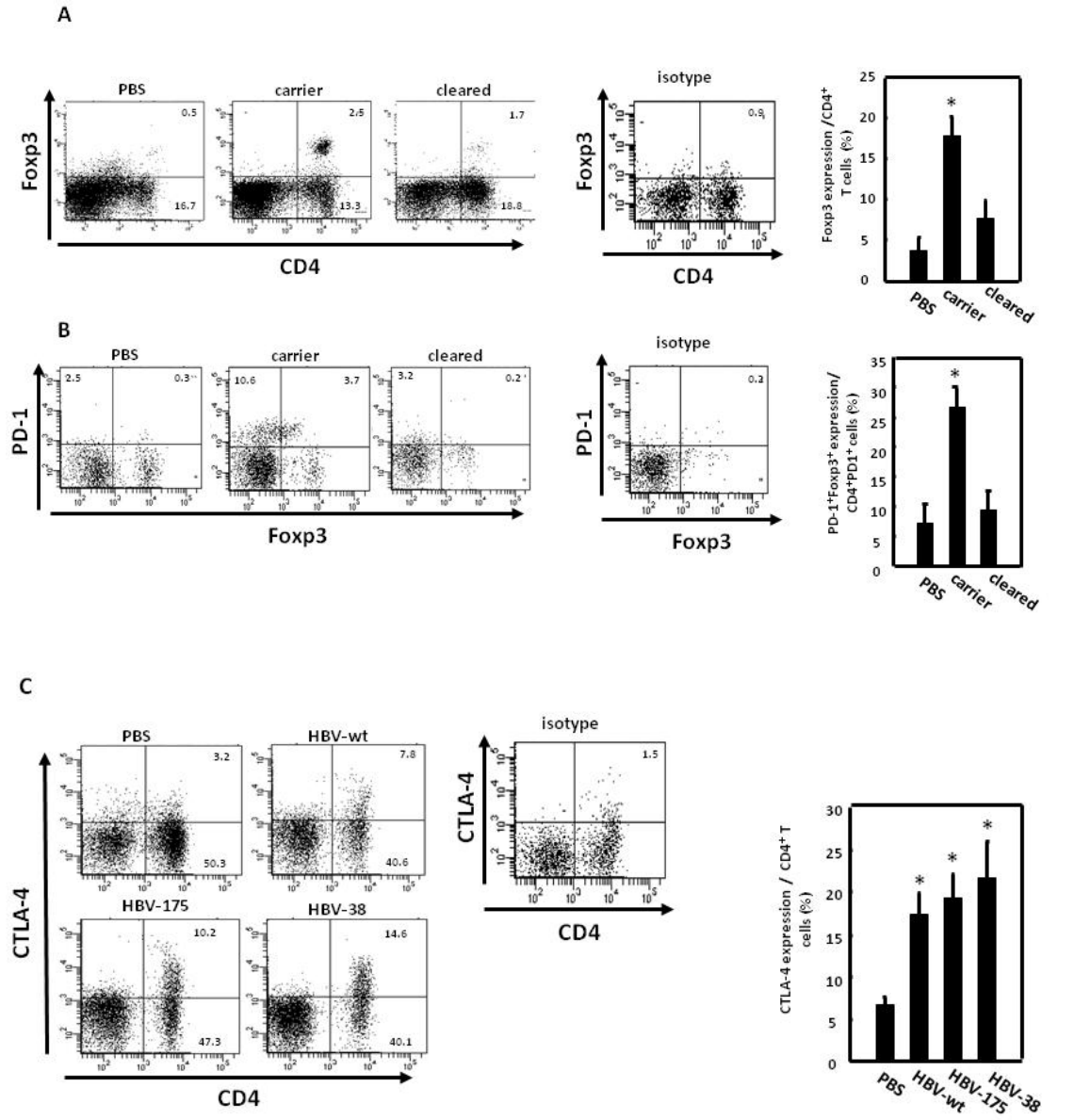


Figure 4. Liver-infiltrating PD-1^{+}CD4^{+} T-cells in mice with hepatitis B viral (HBV) persistence exhibited the phenotype of regulatory T-cells

Intrahepatic lymphocytes from C57BL/6 mice hydrodynamically injected with wild-type (WT) pAAV/HBV1.2 and HBsAg-positive 4 weeks after the injection were isolated, and the expressions of Fox-p3 (A), PD-1 (B), CTL-A4 (C), and were analyzed

by flow cytometry. Serum HBsAg titers were determined by an ELISA. HBsAg-positive mice were defined as having a signal-to-noise (S/N) ratio of ≥ 2.

* $p<0.05$.

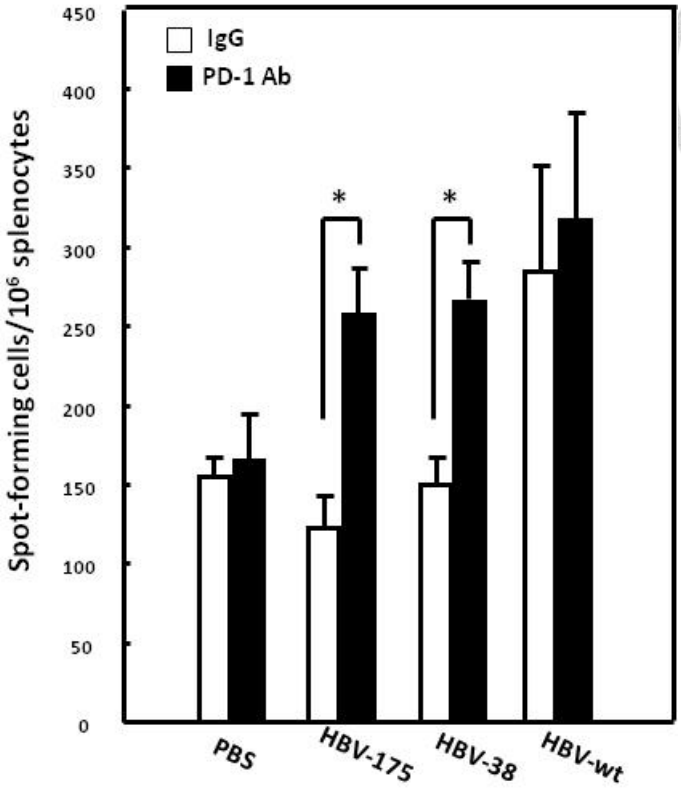


Figure 5. Impaired HBcAg-specific interferon (IFN)-γ production in C57BL/6 mice with hepatitis B viral (HBV) persistence was reversed by treatment of mice with anti-PD-1 mAb

HBcAg-specific IFN-γ responses of splenocytes from mice injected with PBS, wild-type) WT pAAV/HBV1.2 (HBV-wt), HBV-175, or HBV-38 were analyzed by an ELISpot assay. The frequency of HBcAg-specific IFN-γ-secreting cells in the presence of an anti-PD-1 or control antibody were measured. Spot-forming cells per million splenocytes are shown. * $p<0.05$.

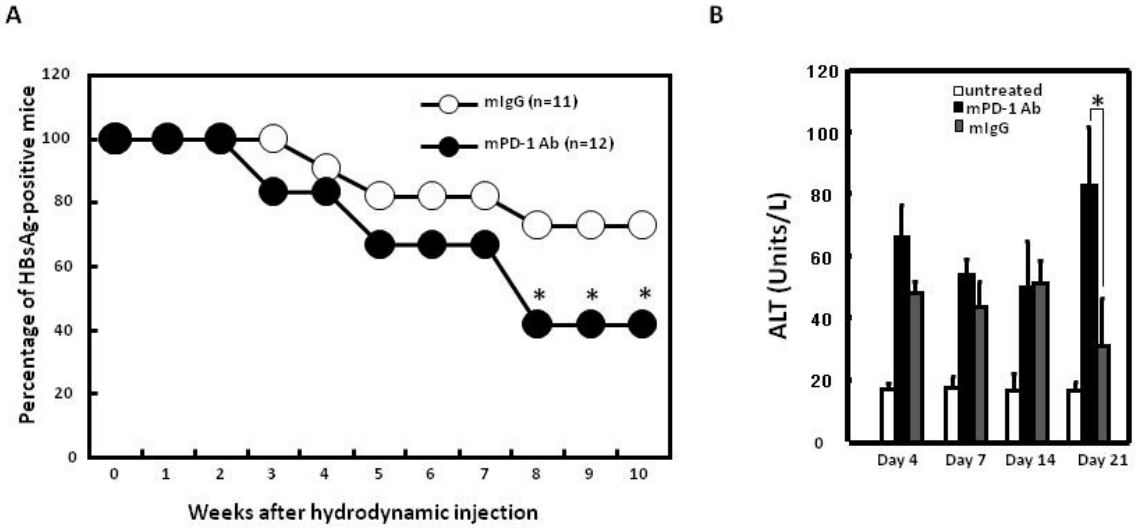


Figure 6. Blockade of the programmed death (PD)-1 pathway by an anti-PD-1 monoclonal antibody (mAb) reduced the hepatitis B viral (HBV) persistence rate in a mouse animal model

C57BL/6 mice were intraperitoneally treated with an anti-PD-1 blocking mAb or isotype control Ab. Antibody administration was initiated every 2 days on day 6 before being injected with an HBV core-truncated (HBV-175) mutant construct. Thereafter, Abs (200 μg) were repeatedly administered every 3~4 days for a period of 8 weeks. (A). The rates of positive serum HBsAg in the mice receiving anti-PD-1 blocking mAb (●, n=12) were compared with those in mice receiving isotype control Ab (○, n=11) (B). The ALT levels in the mice receiving anti-PD-1 blocking mAb, isotype control Ab, and untreated. The ALT level in mice serum was determined by an ELISA. * $p<0.05$.

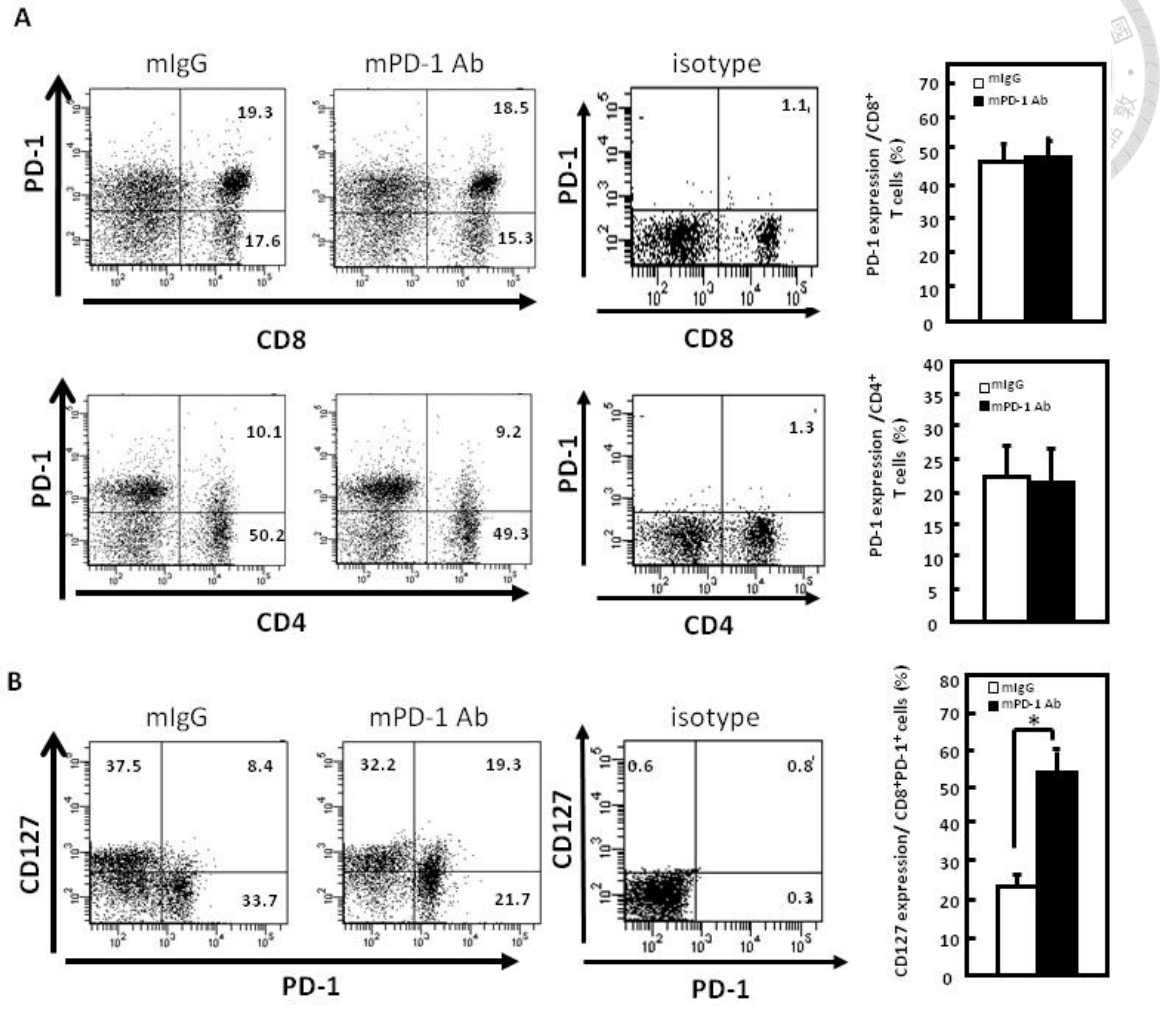


Figure 7. Increased CD127 expression and reversed PD-1^{hi}CD127^{low}-exhausted CD8^{+} T cells phenotype in mice treated with an anti-PD-1 mAb

Intrahepatic lymphocytes from C57BL/6 mice hydrodynamically injected with wild-type (WT) pAAV/HBV1.2 and HBsAg-positive 4 weeks after the injection were isolated, and the expressions of PD-1 (A) as well as coexpression of PD-1 and CD127 (B) on CD4+ and CD8+ T cells in the presence of an anti-PD-1 or control antibody were measured by flow cytometry. * p<0.05 .

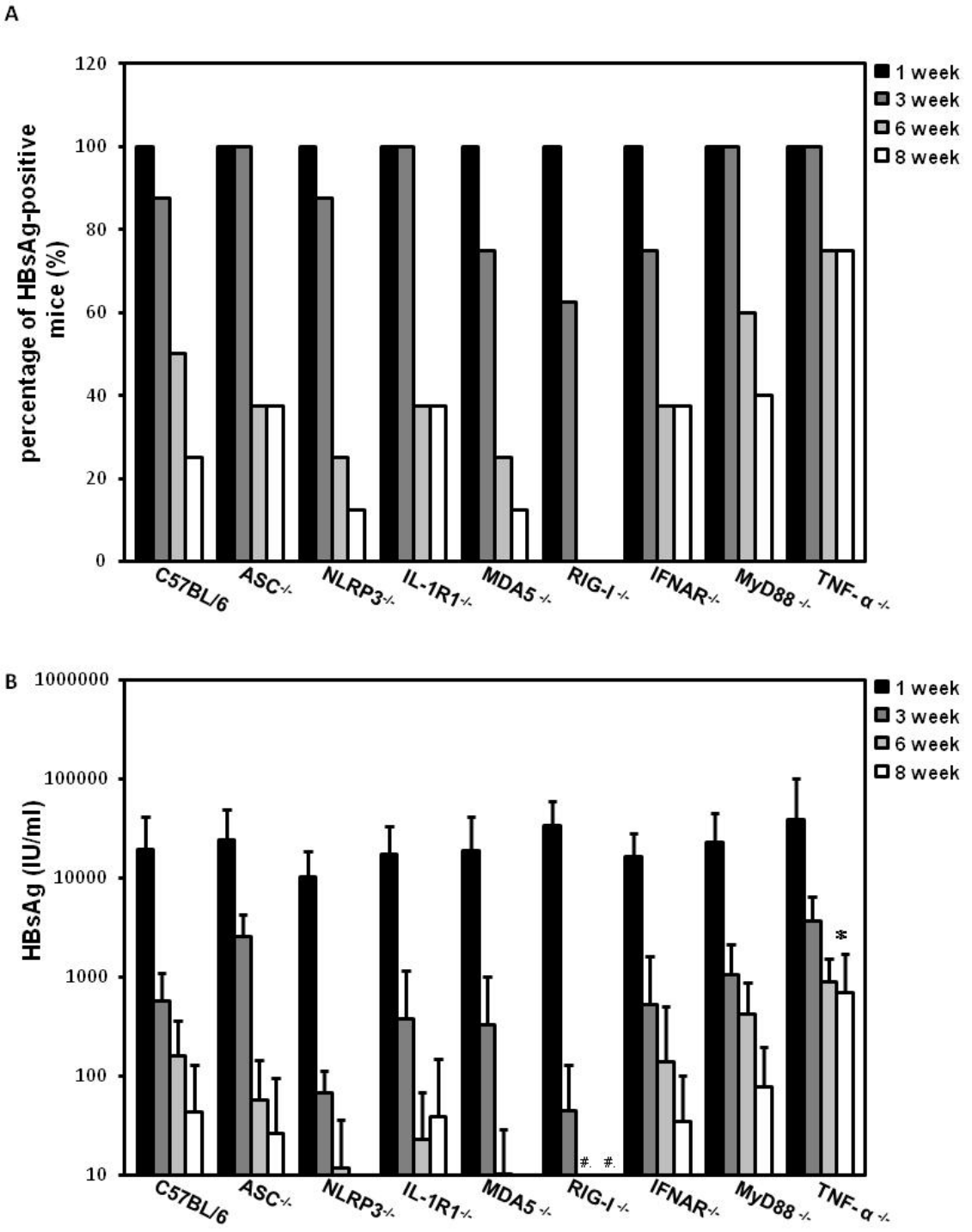


Figure 8. Prolonged HBV persistence in TNF-α knockout mice

Wild type C57BL/6 and different knockout mice, including NLRP3, apoptosis- ASC, MYD88, IL-1R, IFNAR, RIG-1, MAD5, and TNF-α, were introduced to the

pAAV/HBV1.2 plasmid by hydrodynamic injection. HBsAg levels in mice serum were determined by an ELISA. HBsAg-positivity was defined as an S/N ratio greater than 2. The percentage of HBsAg-positivity (A) and serum HBsAg levels (B) were measured at Weeks 1, 3, 6 and 8. #, not detectable.

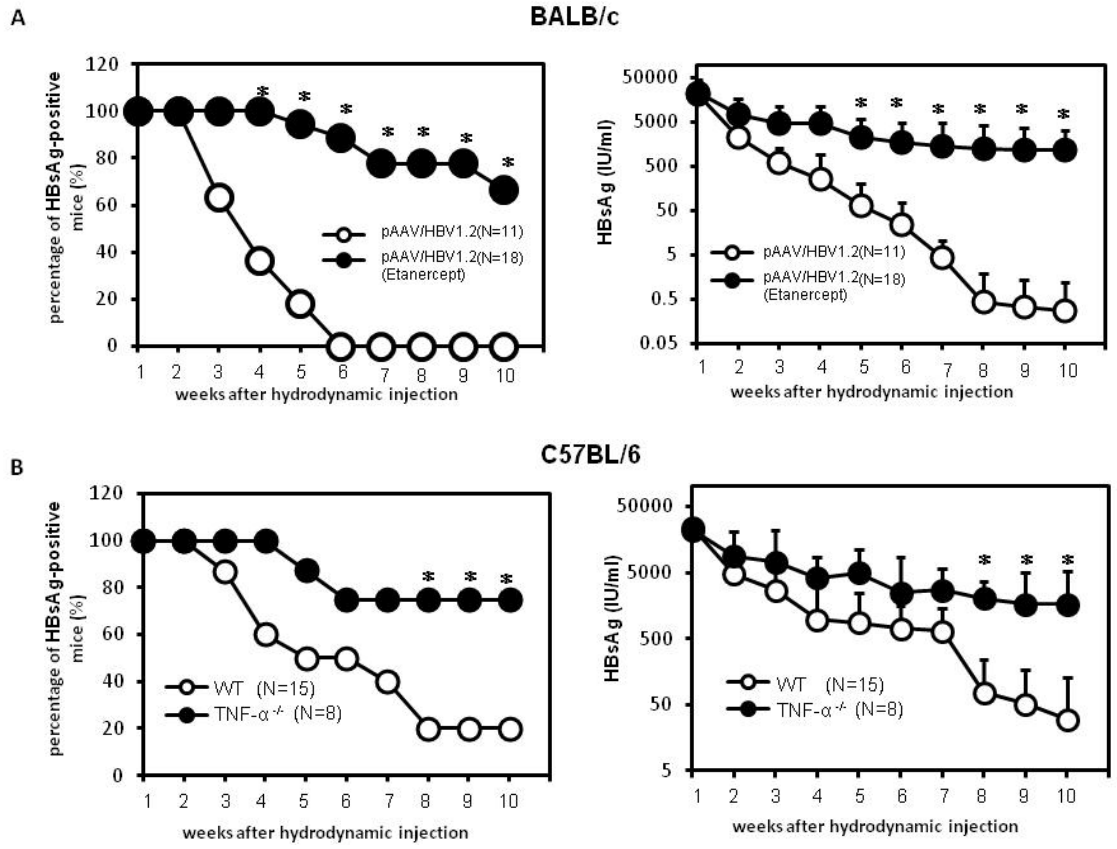


Figure 9. Delayed HBsAg clearance in mice with TNF deficiency

(A) BALB/c mice were treated with the recombinant soluble TNF-α receptor, Etanercept, on the day before hydrodynamic injection of the pAAV/HBV1.2 plasmid. Etanercept treatment was performed twice a week over the detection period. HBsAg levels in mice serum were determined by an ELISA. HBsAg-positivity was defined as an S/N ratio greater than 2. Differences in percentages (left) and serum levels (right) of HBsAg-positive mice with or without Etanercept were quantified. (B) Wild type C57BL/6 or TNF-α knockout mice were hydrodynamically transfected with the pAAV/HBV1.2 plasmid. Differences in percentages (left) and serum levels (right) of HBsAg-positive mice were quantified. * $p < 0.05$.

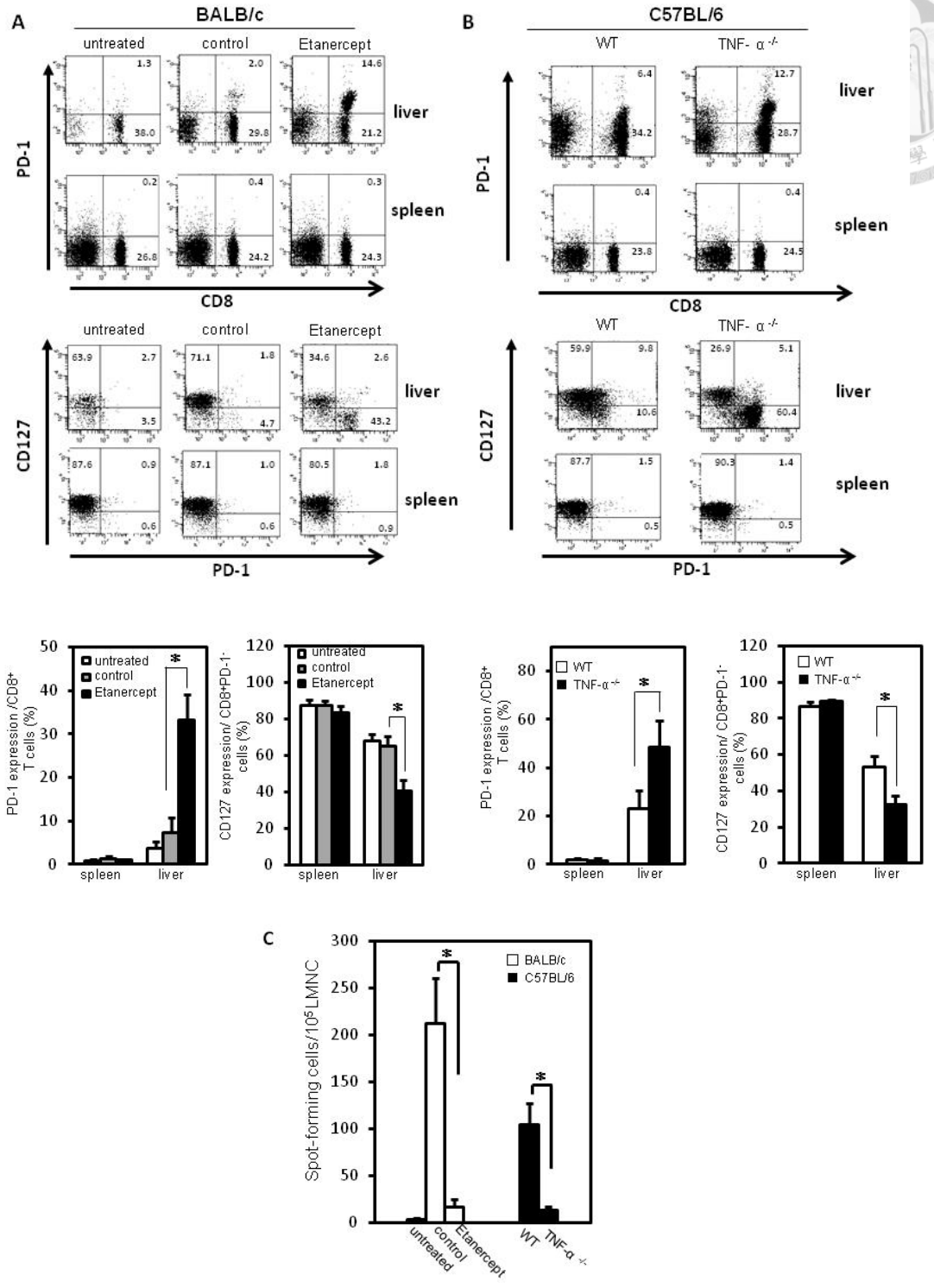


Figure 10. Liver-infiltrating CD8$^+$ lymphocytes in Etanercept-treated and TNF-α knockout mice displayed the PD-1^{hi}CD127^{low}-exhausted phenotype and impaired HBcAg-specific IFN-γ T cell response

(A) BALB/c mice were hydrodynamically injected with WT pAAV/HBV1.2 plasmids in the presence or absence of Etanercept treatment. Eight weeks after injection, intrahepatic lymphocytes from HBsAg-positive mice were isolated and the PD-1, CD127 expressions of liver-infiltrating $CD8^+$ lymphocytes and splenocytes were analyzed by flow cytometry. (B) Wild type C57BL/6 and TNF-α knockout mice were hydrodynamically injected with WT pAAV/HBV1.2 plasmids. Eight weeks after the injection, intrahepatic lymphocytes from HBsAg-positive mice were isolated and the PD-1, CD127 expressions by liver-infiltrating $CD8^+$ lymphocytes and splenocytes were analyzed by flow cytometry. (C) Wild type C57BL/6 and TNF-αknockout mice as well as BALB/c mice with or without Etanercept treatment were hydrodynamically injected with pAAV/HBV1.2. Fourteen days after the injection, liver mononuclear cells (LMNCs) were isolated and HBcAg-specific IFN-γ responses were analyzed by an ELISpot assay. The frequency of HBcAg-specific IFN-γ-secretion was measured as spot-forming cells per 10^5LMNCs. * $p < 0.05$. The data are representative of at least six independent experiments.

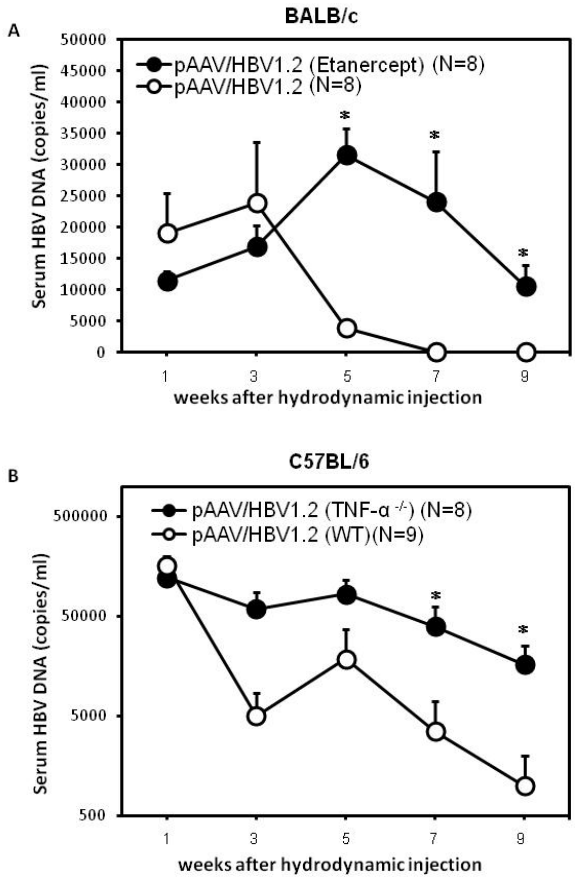


Figure 11. Delayed clearance of serum HBV DNA in mice with TNF deficiency

BALB/c mice with or without Etanercept treatment (A) and TNF-α knockout mice (B) were hydrodynamically injected with pAAV/HBV1.2. The serum HBV DNA in mice was quantified by real-time PCR at the indicated time points. The detection limit for HBV DNA in our system was 1000 copies per milliliter. * $p < 0.05$.

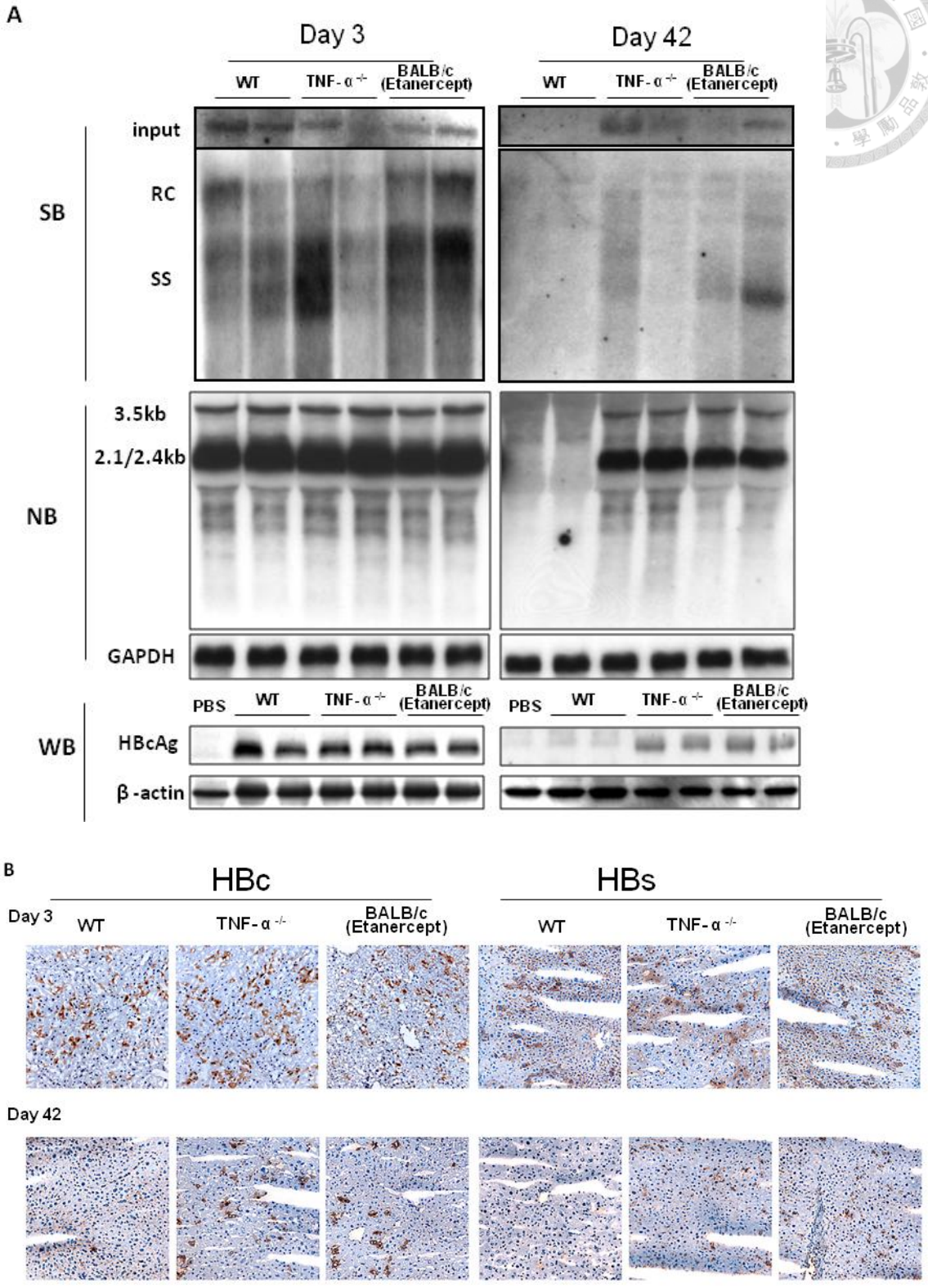
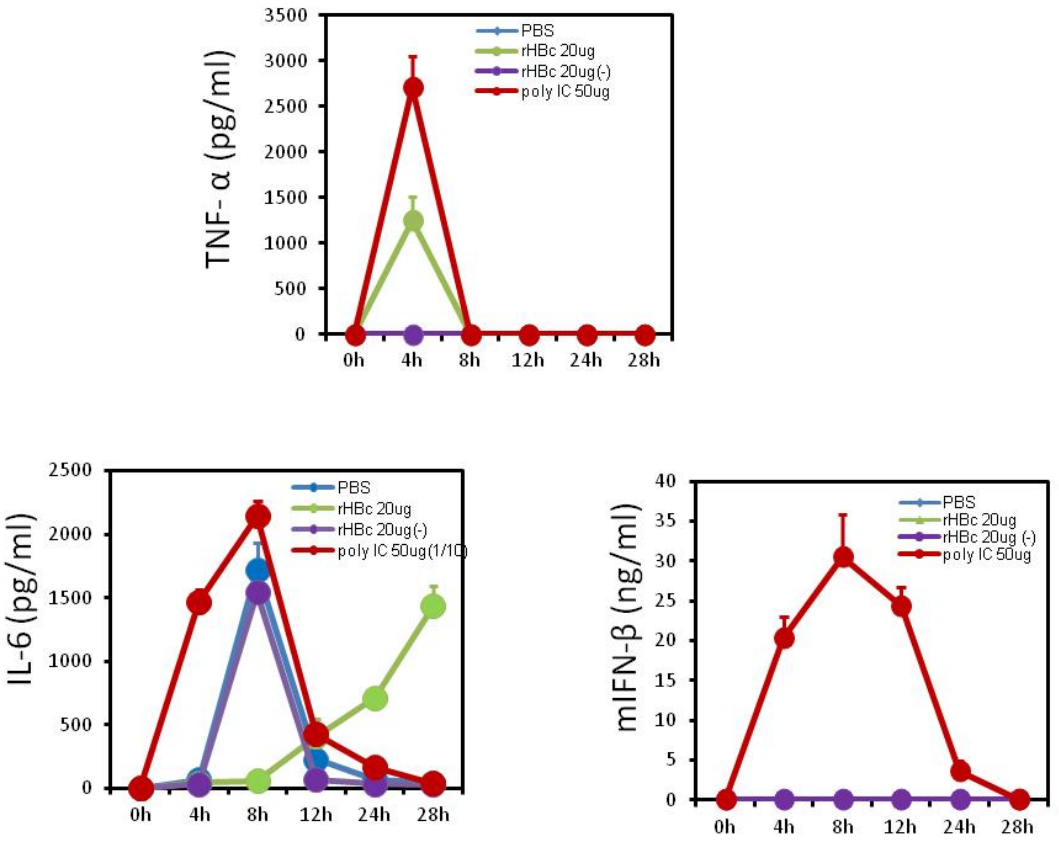


Figure 12. Decreased HBV replication and viral gene expression in Etanercept-treated and TNF-α knockout mice after pAAV/HBV1.2 injection

(A) Liver samples were collected from Etanercept-treated BLAB/c and TNF-α knockout mice on Days 3 and 42 after pAAV/HBV1.2 hydrodynamic injection to examine viral replication, transcription, and HBcAg expression. Intrahepatic HBV DNA and viral transcripts were detected by Southern and Northern blotting, respectively, and GAPDH mRNA was shown as a loading control. The expressions of HBcAg and β-actin (loading control) were analyzed by SDS-PAGE followed by Western blotting. (B) Immunohistochemical staining for expressing HBcAg and HBsAg in the livers of Etanercept-treated BALB/c mice and TNF-α knockout mice compared to C57BL/6 (WT) mice on Days 3 and 42 after pAAV/HBV1.2 injection.



Frigure 13. Hepatitis B viral core capsid triggered innate cytokines production *in vivo*

C57BL/6 mice were divided into four groups and administrated with the indicated reagents, including PBS solution, recombinant HBc, exudates from anti-HBc antibody absorption or poly I:C by hydrodynamic injection, respectively. Serum samples were collected at the indicated time points. The levels of TNF-α (A), IL-6(B), and IFN-β(C) were measured by ELISA kits.

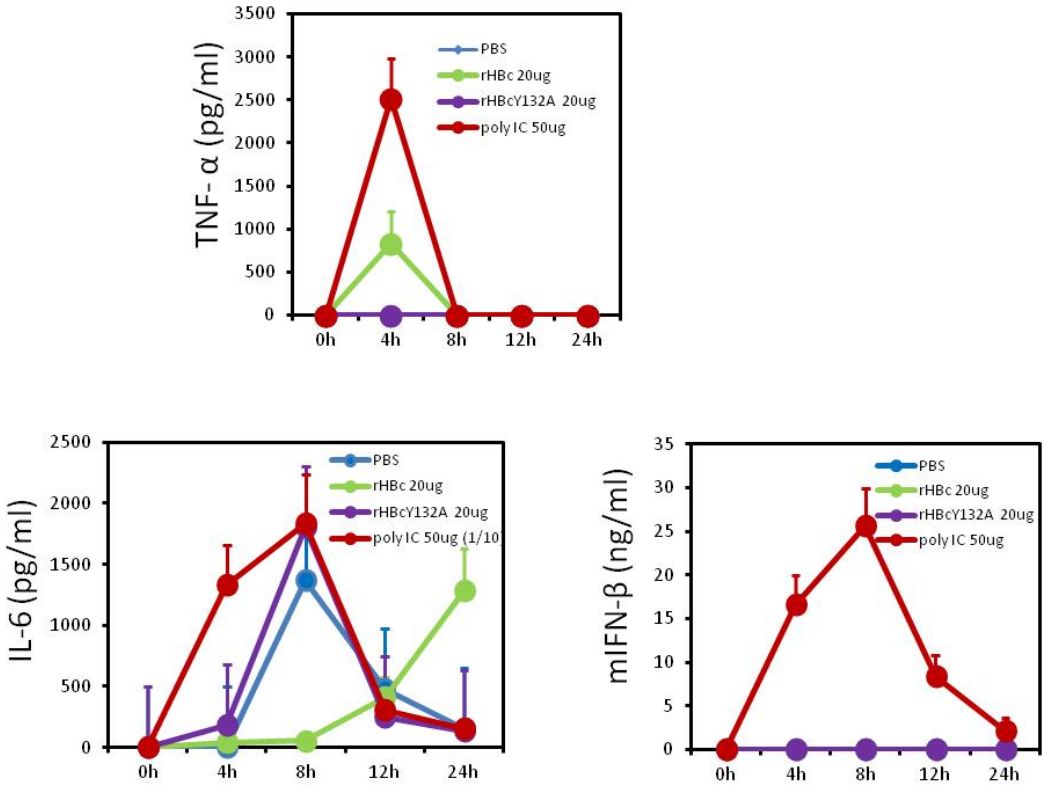


Figure 14. Intact HBV core capsid structure was required for innate cytokines induction

Purified recombinant HBc or assembly-defective mutant HBcY132A were injected into C57BL/6 mice hydrodynamiclly. Mice sera were collected at the indicated time points. The serum levels of TNF-α (A), IL-6 (B) or IFN-β (C) were analyzed by the corresponding ELISA kits.

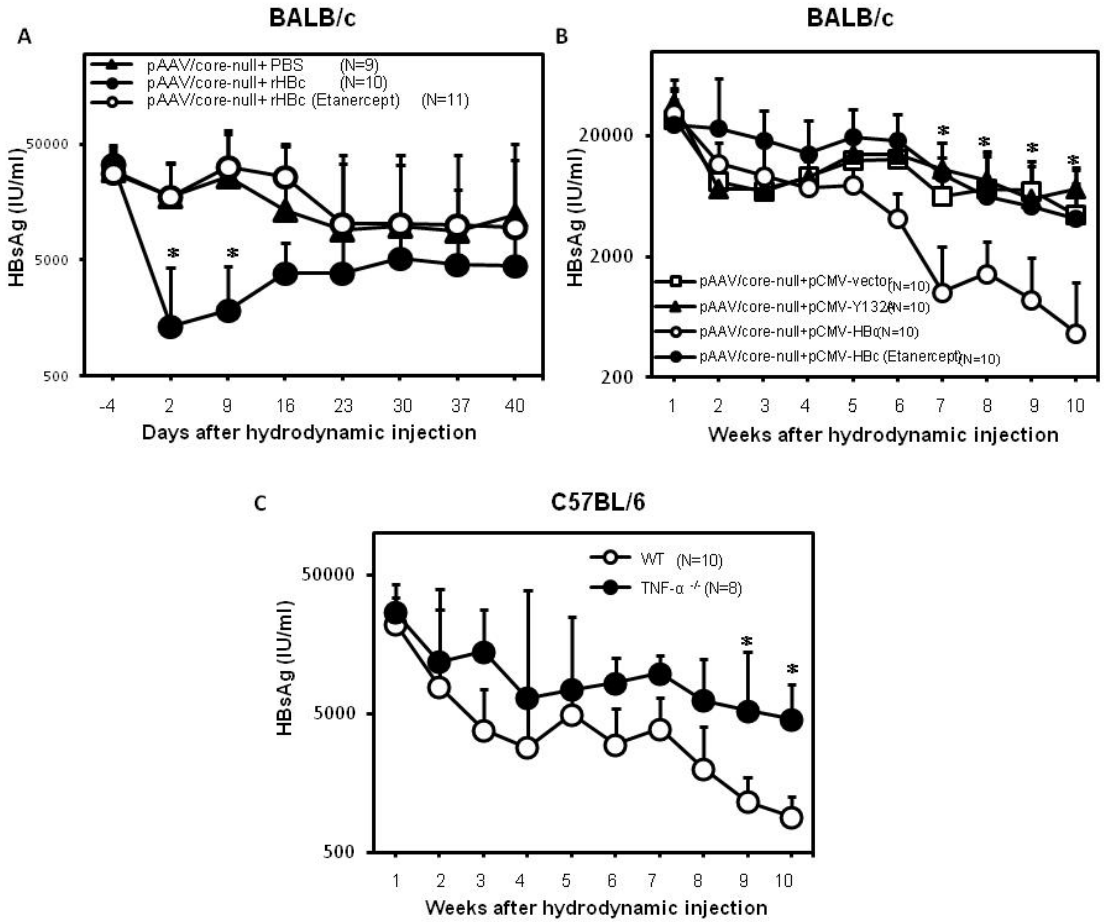
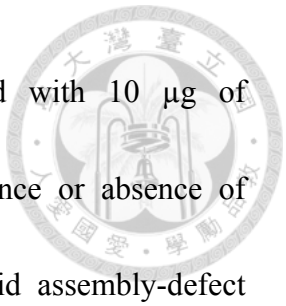


Figure 15. A lack of TNF-α abolished HBcAg-induced HBsAg clearance in mice sera

(A) BALB/c mice were hydrodynamiclly injected with pAAV/e/c-null plasmids, which contain a premature stop codon in the core open reading frame of a replication-competent HBV plasmid pAAV/HBV1.2 on Day -7 in the presence or absence of Etanercept. Etanercept was administered on Days 1, 3, or 5 via intravenous injection. At Day 0, purified recombinant HBV core protein was injected hydrodynamically. The serum HBsAg level was quantified at the indicated time points with an enzyme immunoassay [calculated as IU/ml]. N = number of mice in each



experiment. (B) BALB/c mice were hydrodynamically injected with 10 μg of pAAV/core-null plus 10 μg of pFLAG-CMV2/HBc in the presence or absence of Etanercept or combined with pFLAG-CMV2/HBcY132A, a capsid assembly-defect mutant. The titers of serum HBsAg were measured at the indicated time points. (C) C57BL/6 and TNF-α knockout mice were hydrodynamically injected with 10 μg pAAV/core-null plus 10 μg pFLAG-CMV2/HBc. The titers of serum HBsAg were measured [S/N]. Student's-t test, * $p < 0.05$

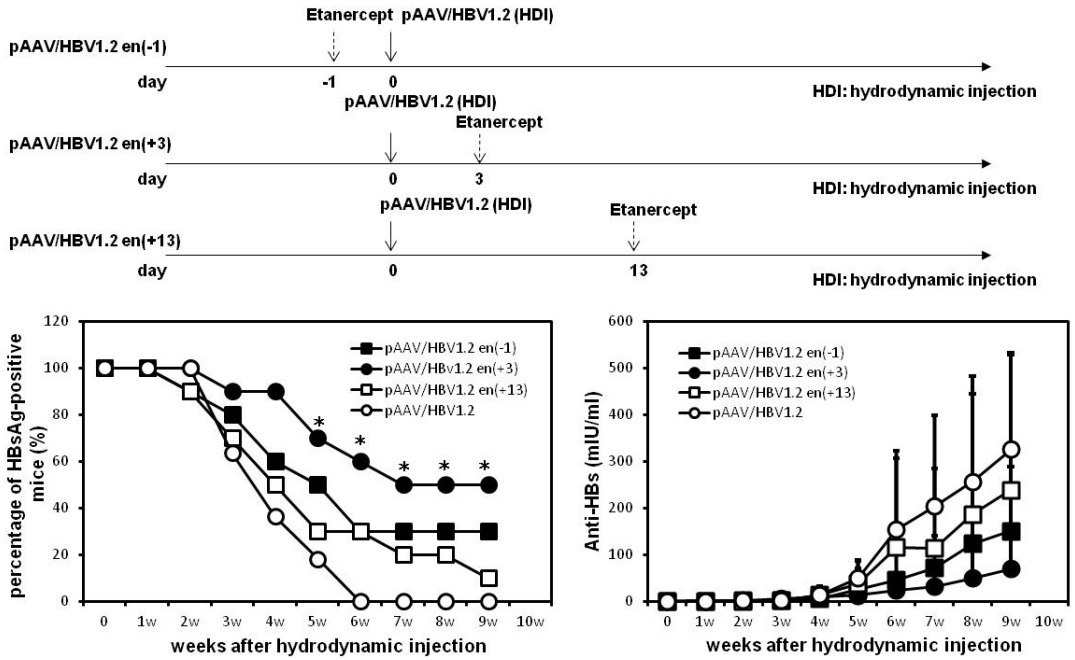


Figure 16. Single dose administration of Enbrel at day 3 postinjection prolonged HBV persistence effectively

BALB/c mice were hydrodynamiclly injected with pAAV/HBV1.2 plasmid at day 0. Single dose of Etanercept was administered intravenously at the indicated time point (day-1, day+3 or day+13 respectively). The serum HBsAg titers were determined weekly with an enzyme immunoassay [calculated as signal/noise (S/N) ratios]. HBsAg positivity was defined as S/N $\geqq$ 2. The serum level of anti-HBsAb (Right) and the rates of positive serum HBsAg (Left) in the mice receiving Enbrel were compared with those in mice receiving pAAV/HBV1.2 plasmid only. *, $p<0.05$

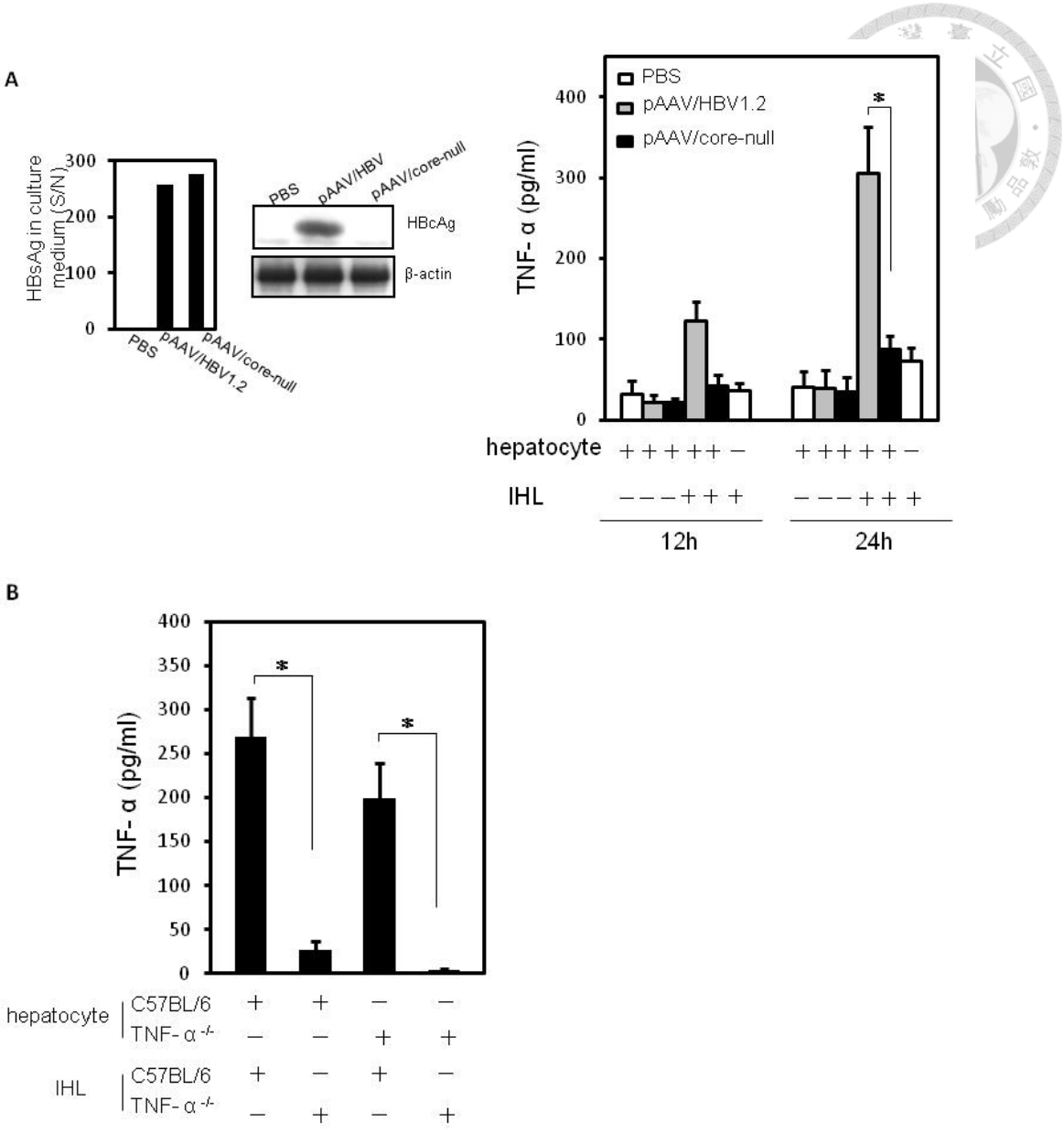


Figure 17. Intra hepatic leukocytes were responsible for HBcAg-induced TNF-α production in an ex vivo culture system

(A). C57BL/6 mice received pAAV/HBV1.2 or pAAV/core-null plasmids by hydrodynamic injection into a tail vein. At Day 3 post-injection, hepatocytes were isolated and cultured for 24 h. The secretory level of HBsAg and intracellular HBcAg expression were determined by enzyme immunoassay (upper left panel) and Western

blotting (upper right panel), respectively. After 24 h of hepatocyte incubation, the intra-hepatic leukocytes were isolated from naïve syngenic mice and co-cultured with hepatocytes for a further 12 or 24 h. The TNF-α production in culture medium was measured by ELISA assay. (B) Wild-type C57BL/6 or TNF-α knockout mice were injected hydrodynamically with pAAV/HBV1.2 plasmids. Hepatocytes were isolated and cultured for 24 h. Naïve intra hepatic leukocytes were isolated from wild-type C57BL/6 or TNF-α knockout mice and incubated with hepatocytes for 24 h. The TNF-α level in culture supernatants was determined by ELISA assay.

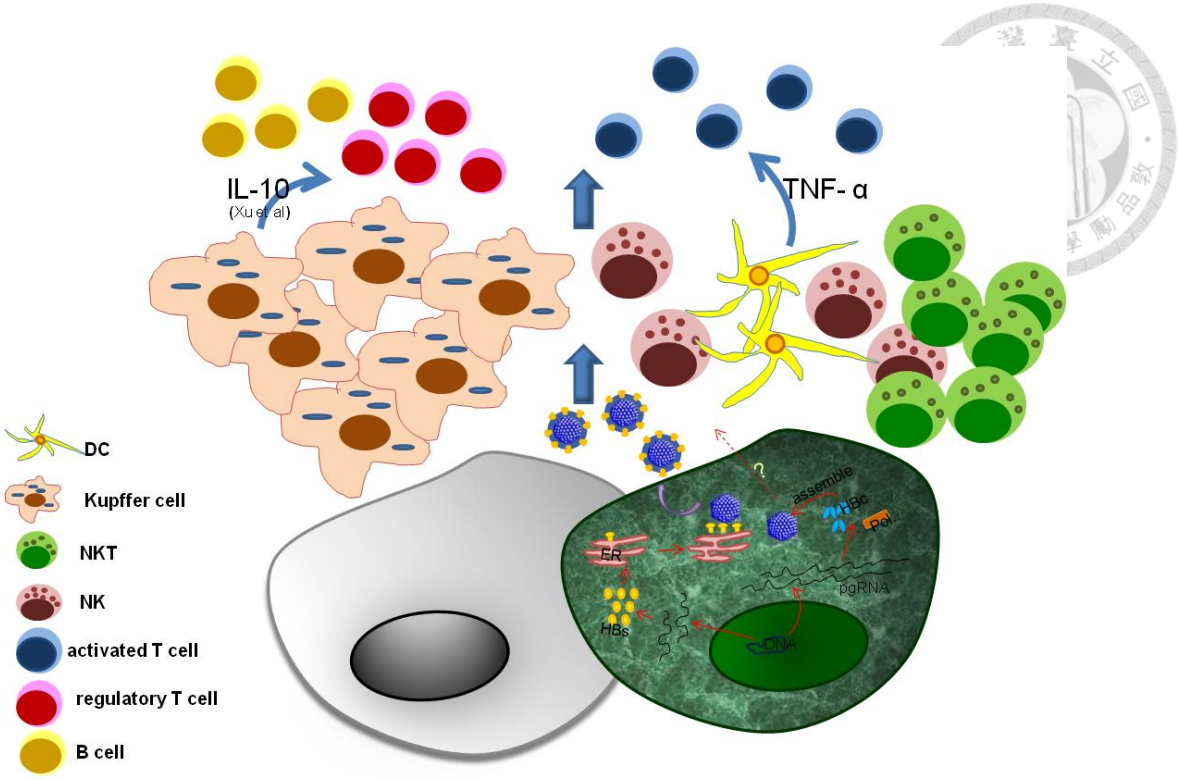


Figure 18. Working hypothesis of HBV-induced immunity in this mouse model

Expression of HBV antigens in hepatocytes induces two arms of immune responses, immune activation and suppression. Icosahedral structure of HBcAg is the critical viral factor for TNF-α release, which is mediated by intrahepatic leukocytes, leading to effective T cell response against HBV. In contrast, HBV also induces Kupffer cells-derived IL-10 production, which promotes the regulatory T cells differentiation and suppresses germinal centers formation, resulting in dampen humoral immune response toward HBV. The interactions between the two arms of immune response which is induced by HBV may provide new insights into the mechanisms of antiviral defense in the liver.

Table 1 Characteristics of mice animal model for studying immune responses toward HBV

	Hydrodynamic-based transfection	Adenovirus delivery	AAV-based transfer
Mechanism to transduce HBV DNA to hepatocytes	physical	Adenoviral receptor	AAV receptor
Procedure-induced innate immunity	damage signals	Proinflammatory	may be Immunosuppressive
HBV-related innate immune responses	not clear	does-dependent	not clear
HBV-related adaptive immune responses	weak to strong	weak to strong	not detected
Factors influencing HBV clearance	viral genetics, host genetics, exposure dosages, environmental factors.	Not known	Not known

Appendix

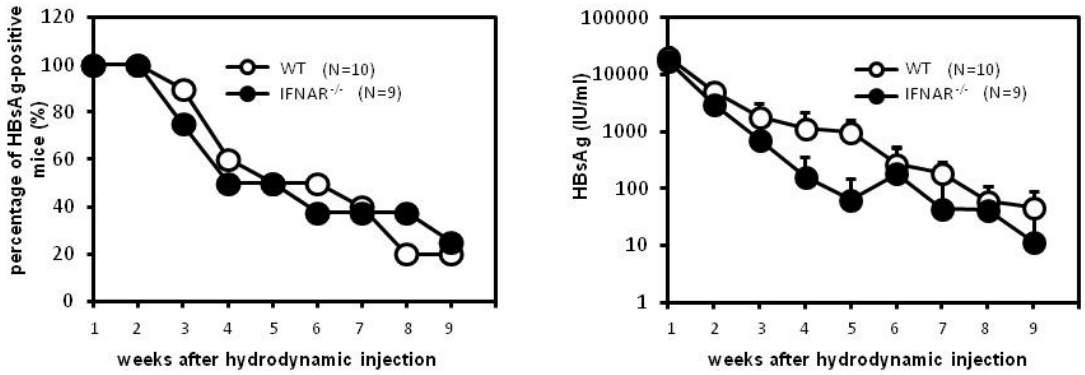


Figure A1. No significant difference in HBsAg clearance between wildtype C57BL/6 and IFNAR knockout mice

C57BL/6 and IFNAR knockout mice were injected with the pAAV/HBV1.2 plasmid hydrodynamically. The serum level of HBsAg was determined weekly via an enzyme immunoassay [calculated as IU/ml]. N equaled the number of mice in each experiment. The positive rate of HBsAg (left panel) and serum HBsAg titers (rifht panel) were shown.

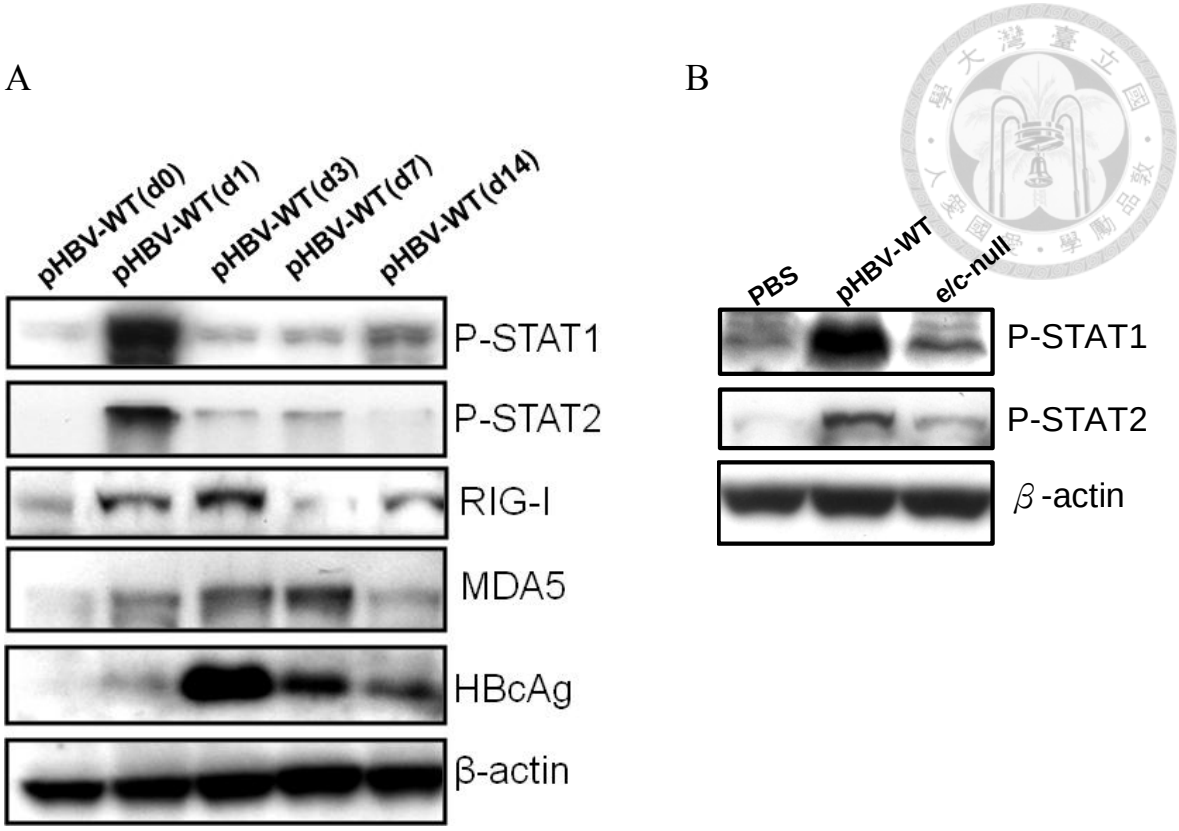


Figure A2. Analysis of the induction kinetics of HBV-responsive genes

(A). C57BL/6 mice were hydrodynamiclly injected with pAAV/HBV1.2 plasmid. At the indicated time points, hepatocytes were isolated and whole-cell lysates were collected for analyzing the erpression of phosphorylated STAT1/STAT2, RIG-I, MDA5 and HBc. (B). C57BL/6 mice were hydrodynamiclly injected with HBV-wt or core-null construct. At day 1 after injection, cell lysates from isolated hepatocytes were collected for analyzing the expression of phosphorylated STAT1/STAT2.

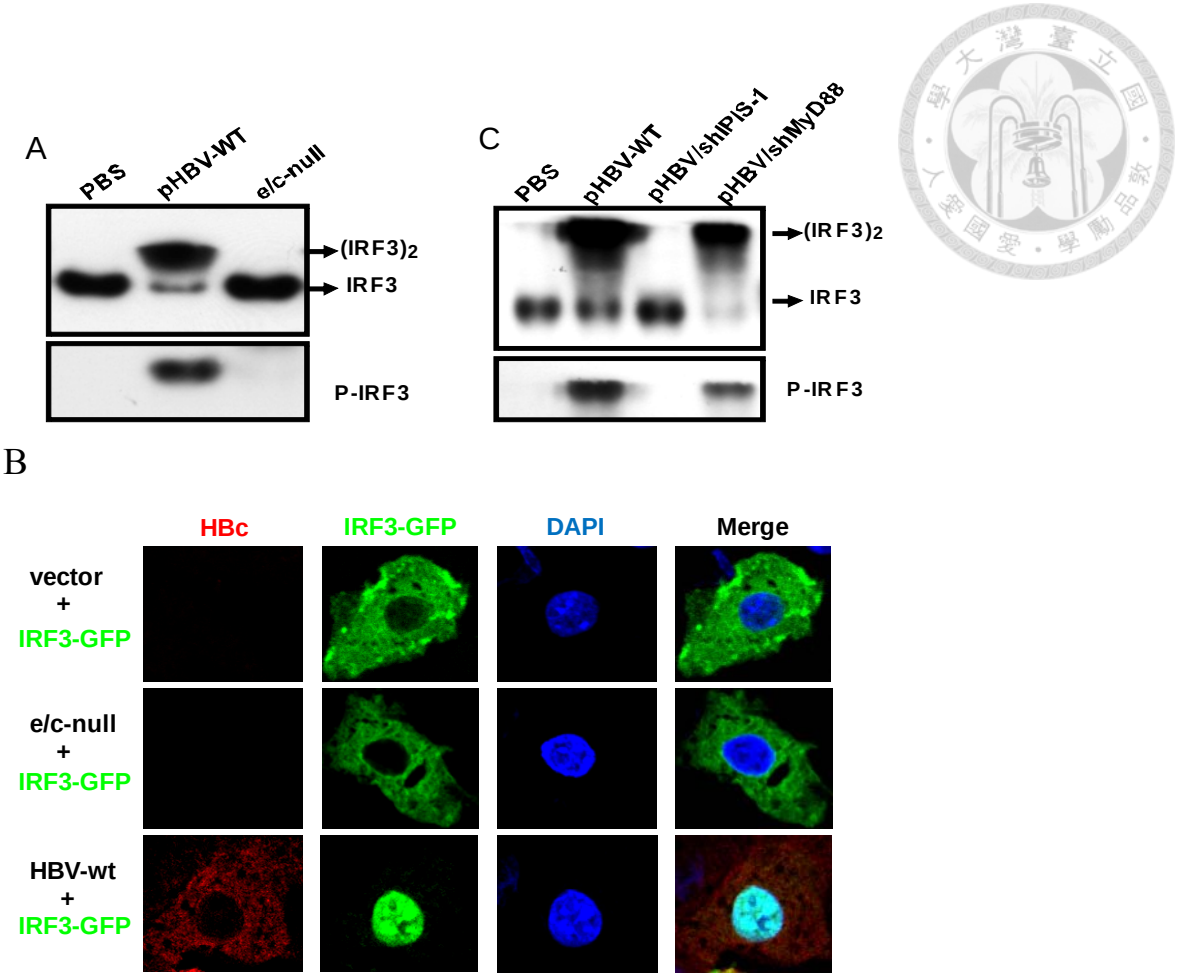


Figure A3. RIG-I-like receptor was critical for HBV core protein-induced IRF3 activation

(A) Hepatocytes extracts were prepared 24 hours after injection of C57BL/6 mice with pAAV/HBV or pAAV/e/c-null constructs. The samples were electrophoresed on native PAGE and analyzed the expression of phosphorylated or dimerized IRF-3 by phosphor-IRF-3 or IRF-3 antibodies. (B) BALB/c mice were hydrodynamicly injected with pAAV/HBV or pAAV/e/c-null plasmids combined with plasmid expressing IRF3-GFP fusion protein. 24 hours after injection, the liver biopsies were collected, and were stained with anti-HBc Ab together with DAPI, and the cellular localization was

determined by confocal microscopy. (C) C57BL/6 mice were injected with pHBV/shRNA constructs, including shRNAs that target MyD88 or IPS-1. At day 3 after injection, hepatocytes were isolated, and the lysates were subjected into native PAGE. The phosphorylated IRF3 or IRF3 dimerization were analyzed by specific antibodies.

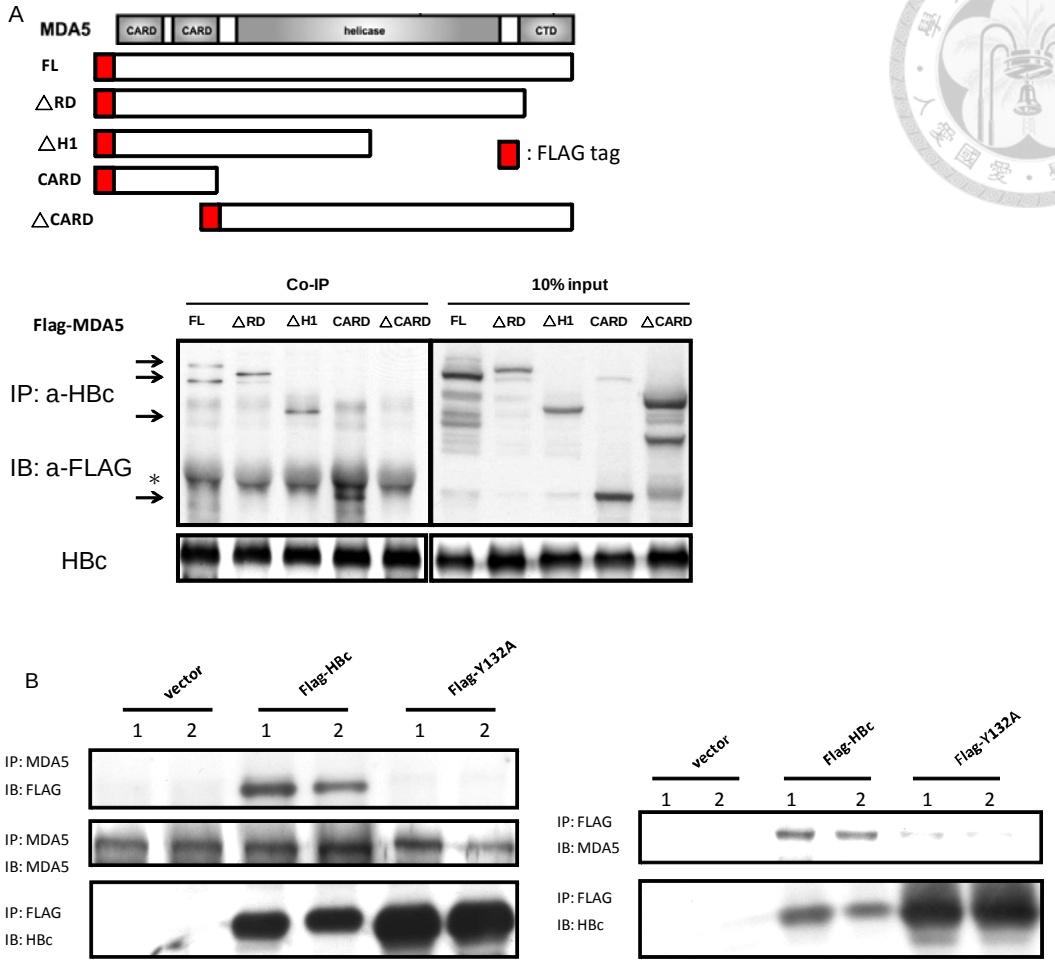


Figure A4. Association of MDA5 and HBV core antigen was dependent on viral capsid structure

(A) Co-immunoprecipitation assays were performed with extracts of 293 cells co-transfected with p-FLAG-HBc plasmid and plasmids expressing wild-type or the mutant MDA5 protein. 293 cell extracts were subjected to immunoprecipitation with anti-HBc antibody, and the immunocomplex were separately immunoblotted with anti-FLAG antibody. (B) BALB/c mice were hydrodynamicly injected with p-FLAG-HBc plasmid or assembly defective mutant (p-FLAG-HBcY132A). 24 hours

after injection, lysates from isolated liver extracts were subjected to immunoprecipitation with anti-MDA5 (left panel) or anti-FLAG antibody (right panel), and the immunocomplex were separately immunoblotted with anti-FLAG or anti-MDA5 antibodies.