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Master Thesis

以 B 型肝炎病毒小鼠模式探討自然殺手 T 細胞的角色

The role of NKT cells in the mouse model of hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a common infection, which can lead to both acute and chronic liver diseases. HBV infection is a major global health problem and chronic viral infection will lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma. However, although the adaptive immunity is essential for viral clearance and controlling HBV infection, the early innate immunity is hard to demonstrate and the innate immune cells involved in HBV clearance are still poorly defined. Recently, results from animal and human studies suggest early response of natural killer T cells (NKT cells) is involved in HBV infection. However, the role of NKT cells in HBV clearance is still not clear. For further exploration of the role of NKT ells in HBV infection, in this study, we use $CD1d^{-/-}$ mice —which are deficient of CD1d-restricted NKT cells— to study the role of NKT cells against HBV in a mouse animal model with hydrodynamic injection approach. Our results demonstrated that in the $CD1d^{-/-}$ mice, there was a higher HBV-positive rate with prolonged HBV persistence. In addition, the CD1d knockout mice were not able to develop protective antibody compared to wild type C57BL/6 mice. Furthermore, there was increased exhausted phenotype of $CD8^{+}$ T cells in liver infiltrating lymphocytes at late phase of HBV transfection, which demonstrated the immune dysfunction of T cells and lead to

impairment of capacity in HBV viral clearance. Thus, our study suggests that NKT cells are involved in the clearance of HBV and may play a role in the development of sufficient adaptive immune responses to HBV infection.

中文摘要

B 型肝炎是個常見的感染並且會引起肝臟急性或慢性的病症。而慢性 B 型肝炎的患者會有較高的風險變成肝硬化及肝癌而導致死亡，是全球重要的健康課題之一。雖然後天性免疫反應已經被研究得很清楚，並且已知對於控制 B 型肝炎扮演重要的角色，但是，早期的先天性免疫反應及其參與的細胞仍然不是很清楚。最近，在動物及人類的研究上推測早期自然殺手 T 細胞對於 B 型肝炎是有免疫反應的，但是其角色還不是很清楚。在我們的研究中，我們使用了 CD1d 缺失小鼠，此種老鼠缺失了由 CD1d 限制的自然殺手 T 細胞，並經由高壓注射法使小鼠感染 B 型肝炎病毒來探討自然殺手 T 細胞對於 B 型肝炎扮演的角色。結果顯示，在感染 B 型肝炎病毒後，跟控制組 C57BL/6 小鼠比起來，CD1d 缺失小鼠血清中帶有 B 型肝炎病毒的比率較高，時間也較長，且無法產生保護性抗體。同時，在感染後期，CD1d 缺失小鼠的毒殺性 T 細胞有 exhaustion 的現象，此現象導致 T 細胞毒殺功能受到影響並且無法清除病毒。總結本篇研究，我們推測自然殺手 T 細胞有助於清除 B 型肝炎病毒及發展足夠的後天性免疫反應來控制 B 型肝炎病毒感染。

Chapter 1
Introduction

Background

Hepatitis B is an infectious disease caused by the hepatitis B virus (HBV) which affects the liver and can cause both acute and chronic diseases. And it is a major global health problem because chronic liver diseases will put the people at high risk of death from cirrhosis and liver cancer[1]. Following acute HBV infection, the risk of developing chronic infection varies inversely with age. The reasons why some people become chronic HBV infection but some can successfully clear the virus and develop protective antibodies is still unclear[2, 3, 4]. So, it is crucial to study the pathogenesis of the diseases, the interplay between virus and host factors and components of successful immune responses during HBV infection.

1.1 The liver as a lymphoid organ

The liver gets a dual blood supply from the arterial system and the portal venous blood returning from the intestine, which is rich in microbial products. So, the liver has an unique immunoregulatory functions mediated by local expression of co-inhibitory molecules and immunosuppressive mediators to prevent inadvertent organ damage[5, 6].

However, these tolerogenic properties make the liver an attractive target site for pathogens, such as hepatitis C virus, hepatitis B virus[1].

In a healthy liver, hepatocytes constitute two thirds of the total cell population, and the rest are the non-parenchymal cells such as liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), kupffer cells, liver resident dendritic cells, and lymphocytes[7]. Notably, the liver's lymphocyte population is selectively enriched in natural killer cells (NK) and natural killer T cells (NKT) compared with the circulation and may contribute to viral defense[8, 9, 10, 11, 12].

1.2 Adaptive immune responses toward HBV infection

Recovery from acute HBV infection results in lasting protective immunity that is mediated by neutralizing HBsAg-specific antibodies[13] and by vigorous multi-epitope-specific $CD4^+$ and $CD8^+$ T-cell responses[1, 14]. Notably, animal models and human studies of acute resolving HBV infection have highlighted that CTL (cytotoxic T lymphocytes) -mediated cytotoxicity is important and required for infection control[15, 16]. In contrast, in chronic HBV infection, the most obvious immune deficiency is the depletion of virus-specific CTLs and their functional inactivation. T cell depletion is mostly due to the enhanced susceptibility of these cells to apoptosis[17, 18]. For example, BIM (BCL-2 interacting mediator of cell death)-mediated apoptosis

which is seen in tolerogenic hepatic priming[19], may be promoted by co-inhibitory signals through CTLA4[20] (cytotoxic T lymphocyte antigen) or by TGF-β[21] (transforming growth factor-β) in the persistent of HBV infection.

The few remaining CTLs in chronic infection have functional defects, also termed exhaustion, which has been reported for persistent viral infections[22, 23]. Classically, in the acute infections, the memory CD8 T cells acquire the ability to persist long term without antigen via IL-7 and IL-15-mediated homeostatic self-renewal. But in chronic infections, exhausted $CD8^+$ T cells usually have low expression of CD122 (the β-chain of the IL-2 and IL-15 receptor) and CD127 (the IL-7 receptor α-chain), respond poorly to IL-7 and IL-15 and so failed to acquire the cardinal memory T cell property of long-term survival. In fact, the exhausted $CD8^+$ T cells become antigen-dependent TCR signaling for the long-term survival[22, 24]. A main cause of T cell exhaustion is due to an excess of co-inhibitory signals that outweighs the co-stimulatory signals[25], PD-1, which has been reported in many papers that result in functional defects of T cells in chronic HBV infection[26, 27, 28]. Additionally, higher levels of ligands for PD-1, PDL1, expressed by liver antigen-presenting cells, further promoted T cell tolerance[29].

In summary, the immune escape strategies used by HBV, the depletion or exhaustion of CTLs and the tolerogenic hepatic microenvironment together contribute to the persistence of HBV infection, but why the combination of host and viral factors that

leads to persistent infection rather than the clearance of virus remains to be investigated. Our current knowledge suggests that innate immunity may play a pivotal role in inducing adequate adaptive immunity[30, 31, 32].

1.3 Innate immune responses toward HBV infection

An acute HBV infection of chimpanzees model, which reveals that non-cytolytic, cytokine-mediated pathway could clear most of the HBV DNA from the serum and liver before a detectable adaptive immune responses in the liver[33], indicating the involvement of innate immunity. It is believed that there exists an innate sensor to recognize HBV to induce immune responses. Indeed, retinoic acid-inducible gene-I (RIG-I) was found to have the ability to sense the 5′-ε region of HBV pregenomic RNA and induce type III interferon in human primary hepatocytes[34]. But whether other sensing molecules other than RIG-I are engaged in the activation of innate responses in other cell types such as antigen presenting cells should be further investigated. Until recently, how the innate immunity modulate the early response toward HBV and then the adaptive immunity was still not clear because lacking of appropriate HBV small animal model. In this study, an immunocompetent mouse model for studying the role of NKT cells in HBV infection is used[35]. Due to the inability to infect the mice with HBV directly, a replication-competent HBV plasmid, pAAV/HBV1.2, was injected

hydrodynamically into the tail veins of mice, which mimics the infection of HBV to hepatocytes, and this model has been commonly used for the HBV studies[27, 36, 37]. As reviewed earlier in the study, the liver is enriched in kupffer cells (KC), natural killer cells (NK) and natural killer T cells (NKT), and the role of kupffer cells has been investigated[38] while NK cells and NKT cells were not fully understood. Kupffer cells-derived IL-10 production, which further promotes the $CD4^+$ $Foxp3^-$ type 1 regulatory T (Tr1)-like cells differentiation and inhibit anti-HBV immunity by negatively regulating differentiation of germinal center (GC) B cells, T follicular helper cells (Tfh), or both[36, 38]. And the role of NK cells was proven that if the mice were depleted with NK cells by anti-asialo GM1 (ASGM1), the mice were not able to clear the HBV and became persistent (data not published). NKT cells, which remain controversial toward HBV infection, should further study to find their roles.

1.4 NKT cells

Natural killer T (NKT) cells are a subset of T lymphocytes that bridge the gap between innate and adaptive immunity. These cells express TCRs, but unlike conventional T cells, they recognize lipid-based antigens present by CD1 families by antigen-presenting cells (APCs)[39, 40]. Humans have five CD1 genes, CD1a through e, whereas mice express only CD1d. The most extensively studied is the CD1d-restricted

natural killer T (NKT) cells[41]. Upon activation, they rapidly produce a variety of cytokines, such as interferon-γ (IFNγ), IL-4, IL-10, IL-13, IL-17, 1L-21 and tumor necrosis factor-alpha (TNF-α), activating other immune cells and consequently playing a key role in early responses to infection.

There are two types of CD1d-restricted NKT cells. Type 1 invariant NKT cells which are defined by their invariant TCR-α chain (Vα24-Jα18 in humans; Vα14-Jα18 in mice) and their ability to recognize the glycolipid α-galactosylceramide (αGalCer)[42]. Type 2 noninvariant NKT cells, which express a different and more diverse TCR repertoire than type 1NKT cells and recognize a variety of lipid antigens, including glycolipids and phospholipids[43, 44].

1.5 Correlation between viral control and NKT cell activation

Recently, the results from animal and human studies suggest the early responses of natural killer T cells (NKT cells) toward HBV infection. In HBV-infected human studies, the author has shown the early activation of NK and peripheral natural T cells, a population that phenotypically resemble classical NKT cells, suggesting that the cellular component of innate immune system is able to sense the HBV infection from the beginning. It is noteworthy that natural T cells reached maximal IFN-γ production 2-3 weeks before NK cells[10]. In another study, type 1 NKT cell numbers were shown to

be reduced in the blood of patients who have chronic HBV infection but increased to normal levels with viral control[9]. In woodchuck studies, NK and NKT cells were activated 48-72 hr after HBV infection and hepatic WHV (woodchuck hepatitis virus) DNA level was transiently but significantly reduced[45]. Moreover, a study using adenoviral delivery of a replication-competent HBV genome into mice shows HBV-induced lipid alterations contribute to NKT cell-dependent protective immunity. The author finds that HBV infection of hepatocyte results in upregulation of self-phospholipid antigens phosphatidylwthanolamine (PE) and the secretory phospholipase ($sPLA_2$) enzymes and that lead to activation of NKT cells. It is noteworthy that NKT activation in response to HBV is mediated by hepatocytes and dependent on hepatocyte CD1d. The absence of NKT cells or CD1d or ER-associated transfer of lipids onto CD1d all lead to defective HBV-specific T cell and B cell responses[8]. These studies demonstrate the correlation between NKT cells and viral control.

1.6 Rationale

Whereas the adaptive immunity is essential for controlling the HBV infection, the importance of early innate immune responses is still controversial and the innate immune cells involved are poorly defined. Recently, increasingly studies demonstrate the correlation between NKT cells and viral control, but these effects shown to be short-term and little is known about whether NKT cell can affect the adaptive immune responses toward HBV. In our study, we use a mouse model with hydrodynamic injection approach to further define the role of NKT cells, and how they affect the outcome of HBV infection. Although NKT cells are less abundant in human liver than mice liver, they may have similar role in promoting immunity to HBV infections.

1.7 Specific aims

1. To identify whether NKT cells have role in the immune responses of HBV infection in HDI mouse model

   Approach: transfection of the hepatitis B virus by hydrodynamic injection of HBV plasmid in C57BL/6 and $CD1d^{-/-}$ mice

2. To see whether $CD1d^{-/-}$ mice have $PD\text{-}1^{hi}CD127^{low}$-exhausted phenotype of liver $CD8^{+}$ lymphocyte at late phase of HBV infection

   Approach: ten weeks after HBV transfection, extract liver non-parenchyma cells (intrahepatic leukocytes) and PD-1 and CD127 expressions by $CD8^{+}$ T cells were analyzed by flow cytometry

Chapter 2
Materials and Methods

2.1 Animals

6~8 week old male C57BL/6 mice were obtained from the National Laboratory Animal Center and maintained under specific pathogen-free (SPF) conditions. CD1d knockout mice[46] were obtained from Professor Alice Lin-Tsing, and also maintained under SPF conditions.

2.2 Hydrodynamic injection[35]

Ten micrograms of HBV plasmid DNA in 1X PBS in a volume equivalent to 8% of the mouse body weight and was injected via tail vein within 5 seconds. Plasmid DNA were purified by endotoxin-free plasmid DNA purification kit.

2.3 Isolation of intrahepatic leukocytes

Mice were anesthetized by intraperitoneal injection of Avertin and the chest and abdomen were opened. Liver was perfused by 10ml of PBS and the whole liver was excised. Then the liver was minced by a plunger and passed through a 100μm nylon mesh by washing with HBSS to final volume 50mL. Hepatocytes and large cell clumps

were removed by centrifuge at 4℃ for 2 minutes at 50xg for two times. The supernatants containing intrahepatic leukocytes (IHLs) was descended by 300xg at 4℃ for 10 minutes. Cell pellet then was resuspended in 40% percoll and gently added and layered on top of 70% percoll, then centrifuge at 25℃ for 20 minutes at 1200xg and viable IHLs would be at the 40%/70% percoll interphase. IHLs were washed with 15 mL HBSS and centrifuge at 4℃ for 10 minutes at 300xg. Cell pellet was collected for downstream applications.

2.4 Detection of the HBV surface antigen, DNA, and antibody in serum

Serum levels of HBsAg were determined using Abbott ARCHITECT i1000 kit. Anti-HBs and anti-HBc antibodies were determined using CMIA. HBV DNA was detected by real-time PCR.

2.5 Immunohistochemistry

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

2.6 Flow cytometry

APC-conjugated anti-mouse CD3, PE-Cy7-conjugated anti-mouse CD8, PE-conjugated anti-mouse NK1.1, PE-conjugated anti-mouse PD-1, and FITC -conjugated anti-mouse CD127 were used for flow cytometry.

2.7 Materials

APC anti-mouse CD3 (145-2C11)	Biolegend, San Diego, CA,USA
PE-Cy7 anti-mouse CD8 (53-6.7)	Biolegend, San Diego, CA,USA
PE anti-mouse PD-1 (29F.1A12)	Biolegend, San Diego, CA,USA
FITC anti-mouse CD127 (A7R34)	Biolegend, San Diego, CA,USA
PE anti-mouse NK1.1 (PK136)	Tonbo biosciences, San Diego, CA,USA
Avertin	Sigma, St.Louis, USA
Percoll	GE Healthcare
HBSS	Corning, Arizona, USA
PBS	0.1M Na_2HPO_4, 0.018M KH_2PO_4, 0.8% NaCl, 0.02% KCl, pH7.4

Chapter 3
Results

3.1 Impaired NKT cell development in CD1d$^{-/-}$ mice

We compared the NKT cells from the spleen and liver of CD1d$^{-/-}$ mice with those from the C57BL/6 mice. The number and percentage of NKT cells were significantly decreased in both spleen and liver in CD1d$^{-/-}$ mice compared with the control mice. The percentage of NKT cells in spleen were reduced by nearly 50%. In the liver, the percentage of NKT cells were reduced by nearly 70~80% (Figure 1A). And in particularly, the majority of the decreased NKT cells in the liver are CD4^{+} NK1.1^{+} NKT cells (Figure 1B). The results are same in the published paper[46].

Also, we analyzed the percentage of liver intrahepatic leukocyte subpopulations in CD1d$^{-/-}$ mice and C57BL/6 mice and found that despite the decreased number of NKT cells in the CD1d$^{-/-}$ mice, the CD4 T cells were also decreased (Figure 2A). And figure 2B are the values represent mean±SD of figure 2A.

3.2 NKT cells are essential for the clearance of HBV and the ability to produce protective antibody anti-HBs

After injection of HBV plasmid, the mice C57BL/6 and CD1d$^{-/-}$ were regularly bled to monitor the serum levels of HBsAg, anti-HBs and anti-HBc. In CD1d$^{-/-}$ mice, there was a higher HBsAg level, HBV-positive rate and prolonged HBV persistence while C57BL/6 was able to clear the HBV and can't detect any HBsAg in serum from week6 (Figure 3A and 3B). In summary, the serum HBsAg levels and HBV persistence rate were enhanced in CD1d$^{-/-}$ mice compared to wild-type C57BL/6 mice. The levels of anti-HBs in serum were also analyzed. CD1d$^{-/-}$ mice were difficult to produce protective anti-HBs compared to wild-type C57BL/6 mice (Figure 4A). These results tell us that NKT cells have roles for the HBV clearance and are essential for the ability to produce protective antibody anti-HBs.

3.3 Immunohistochemistry staining of HBsAg and HBcAg were higher in the livers of CD1d$^{-/-}$ mice

CD1d$^{-/-}$ mice showed elevated viral antigens in the serum compared with the wild type C57BL/6 mice. We then further confirmed whether the viral antigens in the liver have the similar results. As expected, immunohistochemistry analysis also revealed that the staining of HBsAg and HBcAg in the livers of CD1d$^{-/-}$ mice were still detectable on

day 35 post HBV transfection while in the wild type C57BL/6 mice these antigens were much lower (Figure 5 and Figure 6). These results were correlated with the serum viral loads' data.

3.4 PD-1 (programmed death-1) is upregulated in liver-infiltrating $CD8^+$ T cells from $CD1d^{-/-}$ mice with hepatitis B virus transfection

Wild type C57BL/6 and CD1d knockout mice were injected hydrodynamically with HBV plasmid or PBS. Ten weeks post-injection, intrahepatic leukocytes were isolated and PD-1 expression by $CD8^+$ T cells were analyzed by flow cytometry.

PD-1, which has been reported in many papers that results in functional defects of T cells in chronic virus infection[26, 27, 28]. The results (Figure 7) show that PD-1 is highly upregulated in $CD8^+$ T cells in $CD1d^{-/-}$ mice compared with the C57BL/6 mice after HBV transfection and is consistent with the previous data that $CD1d^{-/-}$ mice have a higher HBsAg level which means it's not able to clear the virus.

3.5 Liver-infiltrating $CD8^+$ lymphocytes in $CD1d^{-/-}$ mice displayed the $PD\text{-}1^{hi}CD127^{low}$-exhausted phenotype

Patients with chronic HBV infection express PD-1 positive CD8 T cells also displayed lower levels of the interleukin-7 receptor, CD127, which defined as the exhausted

phenotype[47, 48]. To find whether CD1d$^{-/-}$ mice were also expressing the exhausted phenotype of CD8^{+} T cells after HBV transfection, both CD1d$^{-/-}$ mice and C57BL/6 mice were hydrodynamically injected with the HBV plasmid or the PBS. Ten weeks post-injection, intrahepatic leukocytes were isolated and both PD-1 and CD127 expressions by CD8^{+} T cells were analyzed by flow cytometry. The results show that CD8^{+} lymphocytes in CD1d$^{-/-}$ mice displayed much lower CD127 phenotype compared with the C57BL/6 mice after HBV transfection (Figure 8).

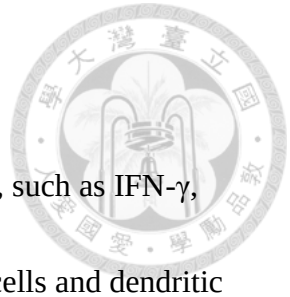
Chapter 4
Discussion

4.1 Defects of adenoviral delivery of the HBV genome

Recently, animal and human studies shed light on the early responses of natural killer T cells toward HBV infection. Though one recent report also investigates the role of NKT cells toward HBV infection[8], the system they used is adenoviral delivery of a replication-competent HBV genome, which is quite different from us. Although adenoviral vectors are efficient vehicles to transfer target genes into the hepatocytes[49, 50], both the adenovirus and the target products can induce the immune responses. Adenovirus infection not only induces proinflammatory cytokines and chemokines[51, 52, 53], but also induces type I IFN *in vivo*[54]. The adenovirus-induced type I IFN is critical in both innate and adaptive immune responses against adenoviral infection and that blockade of type I IFN diminishes innate and adaptive immune responses to adenovirus, leading to the reduction of inflammation and the more stable transgene expression *in vivo*. So, since the use of empty adenoviral vectors can cause both the innate and adaptive immune responses in the liver, it's uncertain that the immune responses seen in this system is caused by the HBV or the adenovirus.

4.2 NKT cells are involved in the development of sufficient adaptive immune responses to HBV infection

In our system, after hydrodynamically injection of HBV plasmid into C57BL/6 and $CD1d^{-/-}$ mice, we found that the clearance rate of HBV antigen were significantly slower in the $CD1d^{-/-}$ mice, though after four months their serum HBsAg were all undetectable. The levels of anti-HBs in serum were also analyzed; we found that $CD1d^{-/-}$ mice were difficult to produce protective anti-HBs compared to wild-type C57BL/6 mice. And there were increased exhausted phenotype of $CD8^{+}$ T cells in liver infiltrating lymphocytes at late phase of HBV infection in $CD1d^{-/-}$ mice, which demonstrated the immune dysfunction of T cells. It raises an important question here that one paper points out that following the resolution of acute infection from HBV, the HBV is controlled but not completely eliminated, and may reactivate under immunosuppression. And strong CTL response will help keep the virus under control[55]. In our studies, although we couldn't detect HBV antigen in serum four months after transfection of HBV to $CD1d^{-/-}$ mice, the failure of developing sufficient adaptive immune responses include anti-HBs antibody and CTL responses should be more concerned.



4.3 The correlation between NK and NKT cells

Upon activation, NKT cells can produce a large number of cytokines, such as IFN-γ, IL-4 and TNF-α and have the ability to activate NK cells, T cells, B cells and dendritic cells[56, 57, 58]. NKT cells are therefore thought to be important for activation of innate immunity and induction of adaptive immunity. In influenza virus infection model, the survival rate of $CD1d^{-/-}$ mice was significantly lower than the wild type mice and with delayed virus clearance in the lungs. Moreover, the cytotoxicity of NK cells and antigen-specific CD8 T cells were impaired in $CD1d^{-/-}$ mice[59]. In another study using murine cytomegalovirus (MCMV) infection model, NK cells were shown to be the major effectors in improved MCMV clearance mediated by α-GalCer therapy[60]. These studies suggest that there is a correlation between NK and NKT cells in some virus infection. In our study, we have found that after NK cell-depleting anti-asialo GM1 antibody treatment, most of the NK cells were depleted but also the percentage of NKT cells in the liver were significantly lower than WT mice. And in anti-asialo GM1 treated mice, there was a higher HBV-positive rate with prolonged HBV persistence after HBV transfection (data not shown). In $NFIL3^{-/-}$ mice, which lack NK cells, whereas the percentage of NKT cells in the liver was significantly higher than WT mice. And after HBV transfection, $NFIL3^{-/-}$ mice clear the HBV more quickly than the WT mice (data not shown). It is of interest to know whether there is a correlation

between NK and NKT cells in HBV infection and further experiments are needed to confirm this question.

4.4 The limitations of using CD1d$^{-/-}$ mice

The roles of NKT cells in antiviral immune responses have been discussed in several virus infection models using CD1d$^{-/-}$ mice[61]. However, CD1d$^{-/-}$ mice lack both type I and type II NKT cells. Hence, it is unable to distinguish whether the phenotype we seen in CD1d$^{-/-}$ mice is due to type I or type II NKT cells or both. Another mouse strain that is also commonly used to investigate the role of NKT cells, Jα18$^{-/-}$ mice, which is specifically deficient in type I NKT cells, can be combined with CD1d$^{-/-}$ mice to distinguish the role between type I and type II NKT cells[62]. However, a published study found that the rearrangements of all the Jα regions upstream of *Traj18* were suppressed and thereby caused substantial distortion of the TCRα repertoire[63]. Fortunately, a recent study has generated a new strain of Jα18$^{-/-}$ mice that lack type I NKT cells while maintaining a complete TCR repertoire[64]. And in our preliminary study using α-GalCer to activate type I NKT (iNKT) cells have found that at day 3 after HBV transfection, the serum HBsAg was significantly lower than untreated mice, showing the possibility that type I NKT cells were able to clear most of the HBV (data not shown). However, to clearly clarify the roles of type I and type II NKT cells,

combined use of Jα18$^{-/-}$ mice and CD1d$^{-/-}$ mice is useful to distinguish between type I and type II NKT cells at the functional level *in vivo*.

Figures

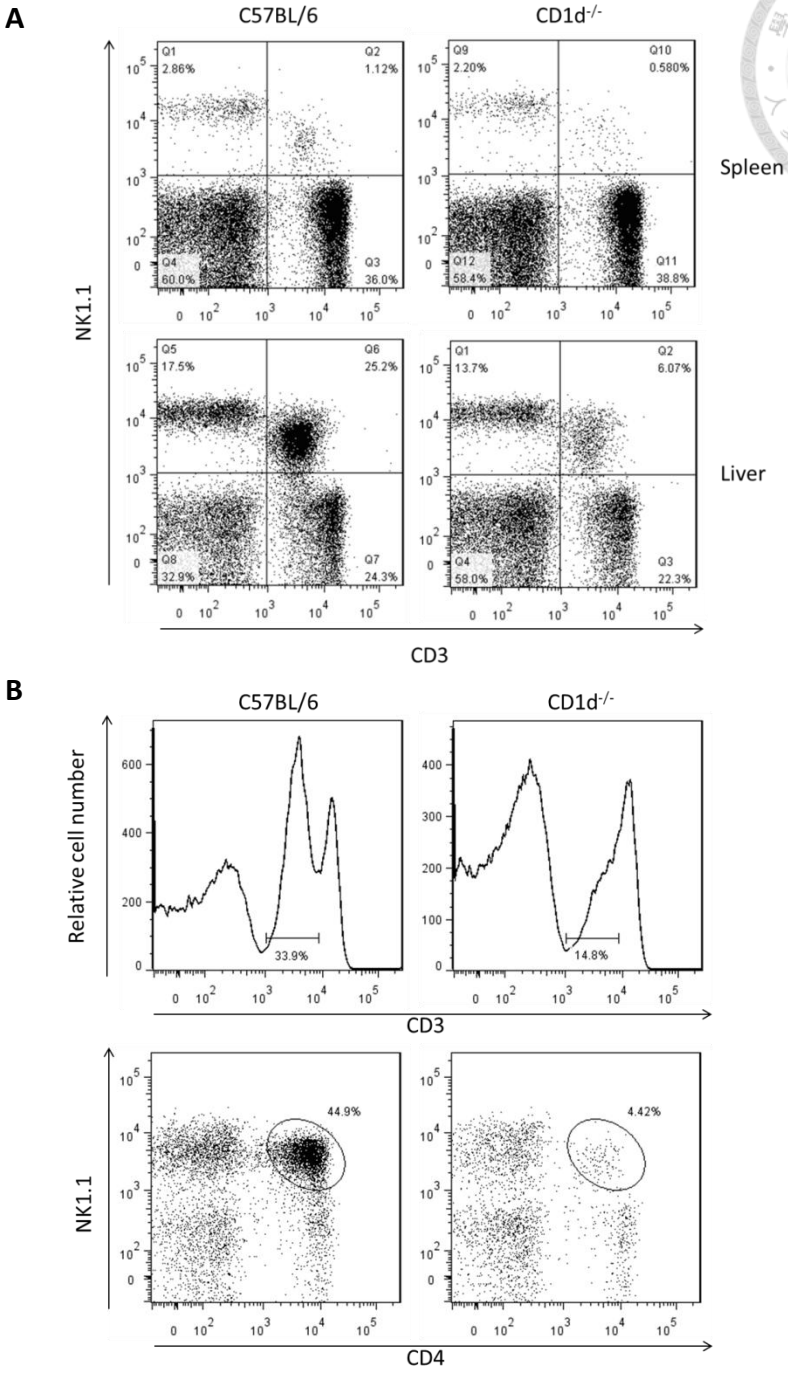
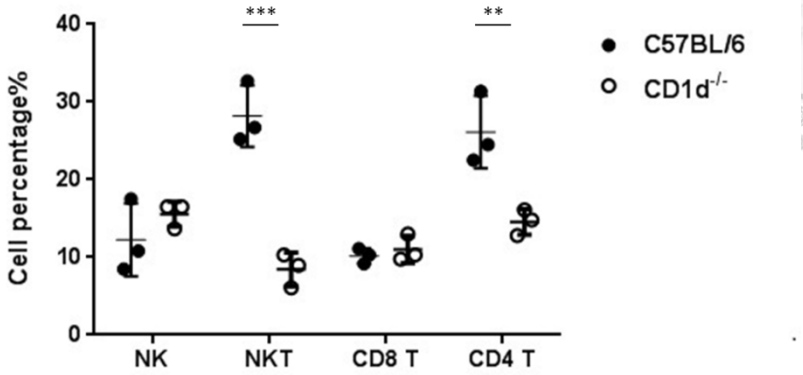


Figure 1 Impaired NKT cell development in CD1d$^{-/-}$ mice

(A) Spleen and liver intrahepatic leukocytes were stained with APC-anti-CD3 and PE-anti-NK1.1 and were analyzed by flow cytometry. The results are representative of 3 C57BL/6 mice and 3 CD1d$^{-/-}$ mice 6-8 weeks old.

(B) Liver intrahepatic leukocytes were stained with APC-anti-CD3, FITC-anti-CD4, PE-Cy7-anti-CD8 and PE-anti-NK1.1 and were analyzed by flow cytometry. The surface expression of CD3 was displayed as a histogram (Top). The numbers represent the percentage of $CD3^{int}$ cells relative to the total population of gated intrahepatic leukocytes. The bottom row of CD4 and NK1.1 staining was gated on $CD3^{int}$ population and the $CD4^{+}$ $NK1.1^{+}$ cells are circled. The numbers represent the percentage relative to $CD3^{int}$ cells. The results are representative of 3 C57BL/6 mice and 3 $CD1d^{-/-}$ mice 6-8 weeks old.

A



B

	Percentage of lymphocytes		
Tissue	Phenotype	C57BL/6	CD1d-/-
Liver	NK	12.3±4.7	15.6±1.6
	NKT	28.2±4	8.5±2.2
	CD4	26.1±4.7	14.6±1.7
	CD8	10.2±1	11±1.7

Figure 2 Percentage of intrahepatic leukocyte subpopulations in CD1d$^{-/-}$ mice and C57BL/6 mice

(A) The percentage of liver intrahepatic leucocytes include NK, NKT, CD4 T cells and CD8 T cells were analyzed by flow cytometry. The results are representative of 3 C57BL/6 mice and 3 CD1d$^{-/-}$ mice 6-8 weeks old.

(B) Values represent mean±SD of figure (A).

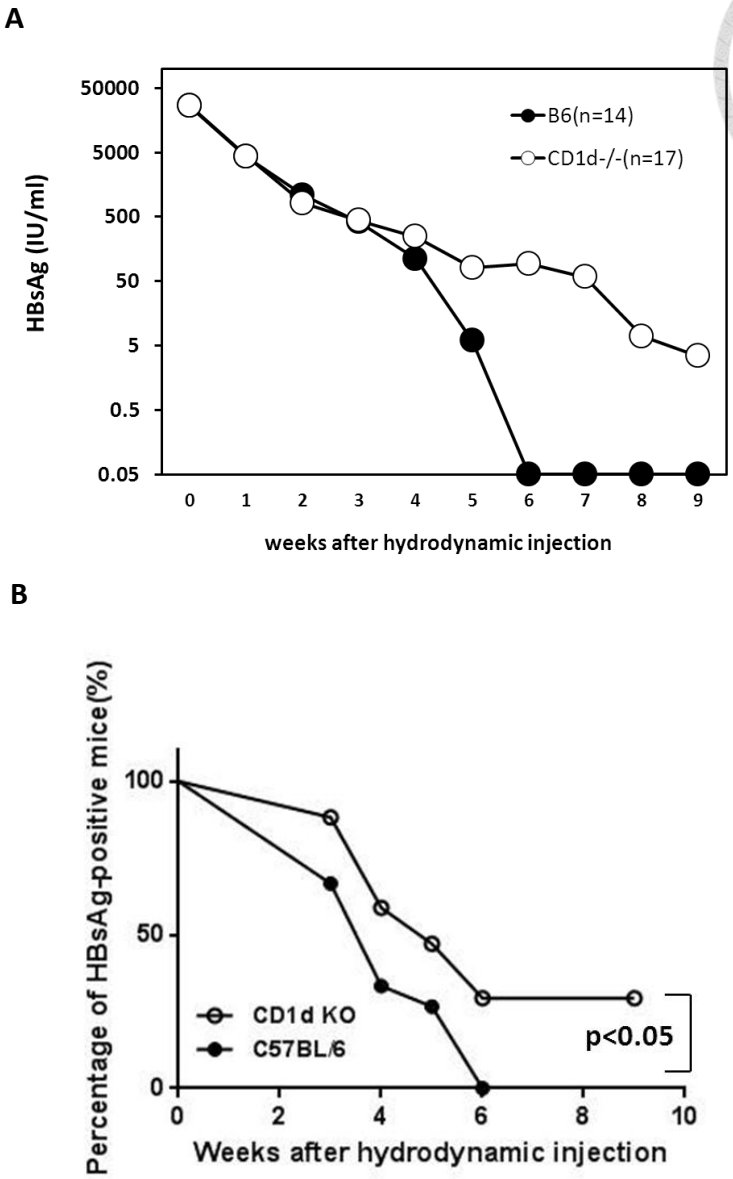


Figure 3 Delayed HBsAg clearance in CD1d$^{-/-}$ mice

Wild type C57BL/6 and CD1d knockout mice were injected hydrodynamically with HBV plasmid. The serum HBsAg levels (A) and percentage of HBsAg-positivity (B) were measured every week. HBsAg-positivity defined as levels greater than 0.05 IU/ml. *P<0.05, **P<0.01

A

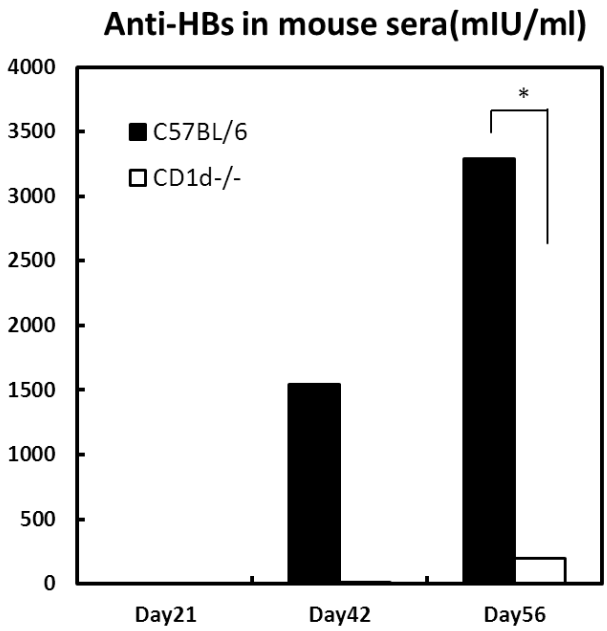


Figure 4 CD1d$^{-/-}$ mice failed to induce production of neutralizing anti-HBs antibodies

In both C57BL/6 and CD1d knockout mice, titers of anti-HBs in their serum were measured every week after hydrodynamically injection of HBV plasmid. The success of producing anti-HBs defined as levels greater than 10 mIU/ml.

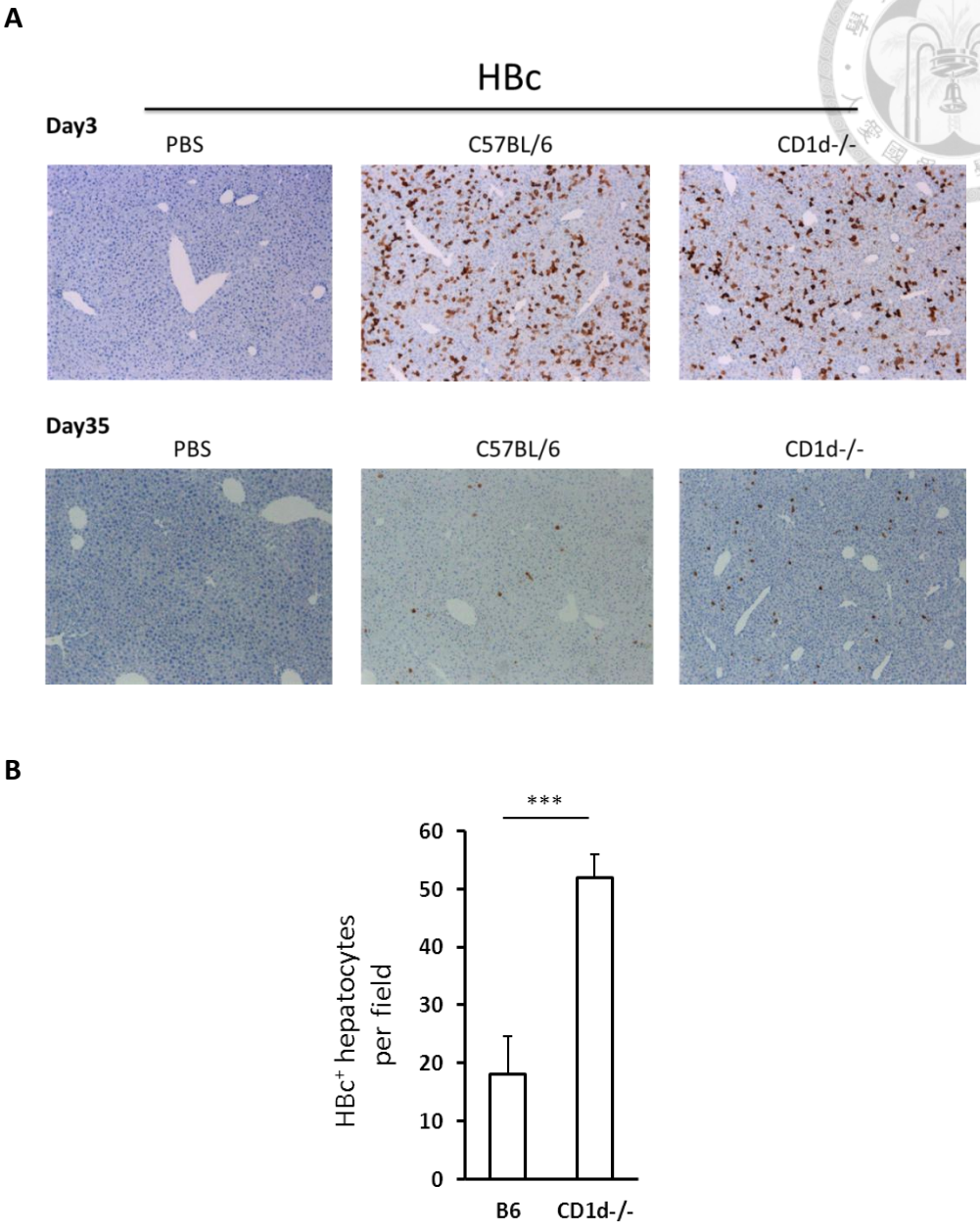


Figure 5 Immunohistochemical staining of HBcAg in the liver of C57BL/6 and CD1d$^{-/-}$ mice after hydrodynamic injection of HBV plasmid

(A) Immunohistochemical staining of HBcAg in the liver of CD1d knockout mice compared to C57BL/6 mice at day3 and day35 after hydrodynamically injection of HBV plasmid. (B) Quantification of HBc^{+} hepatocytes in figure (A) at day35.

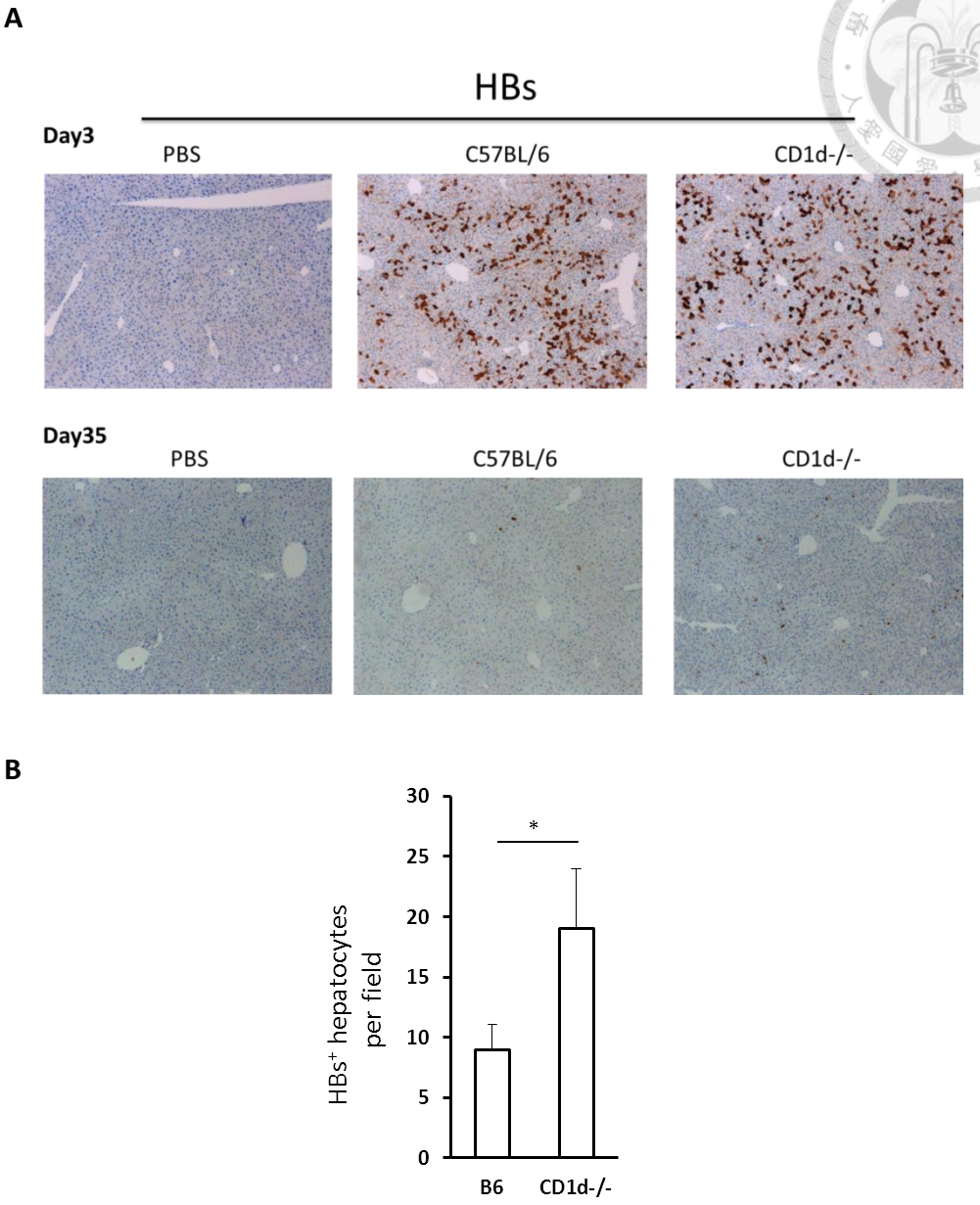


Figure 6 Immunohistochemical staining of HBsAg in the liver of C57BL/6 and CD1d$^{-/-}$ mice after hydrodynamic injection of HBV plasmid

(A) Immunohistochemical staining of HBsAg in the liver of CD1d knockout mice compared to C57BL/6 mice at day3 and day35 after hydrodynamically injection of HBV plasmid. (B) Quantification of HBs^{+} hepatocytes in figure (A) at day35.

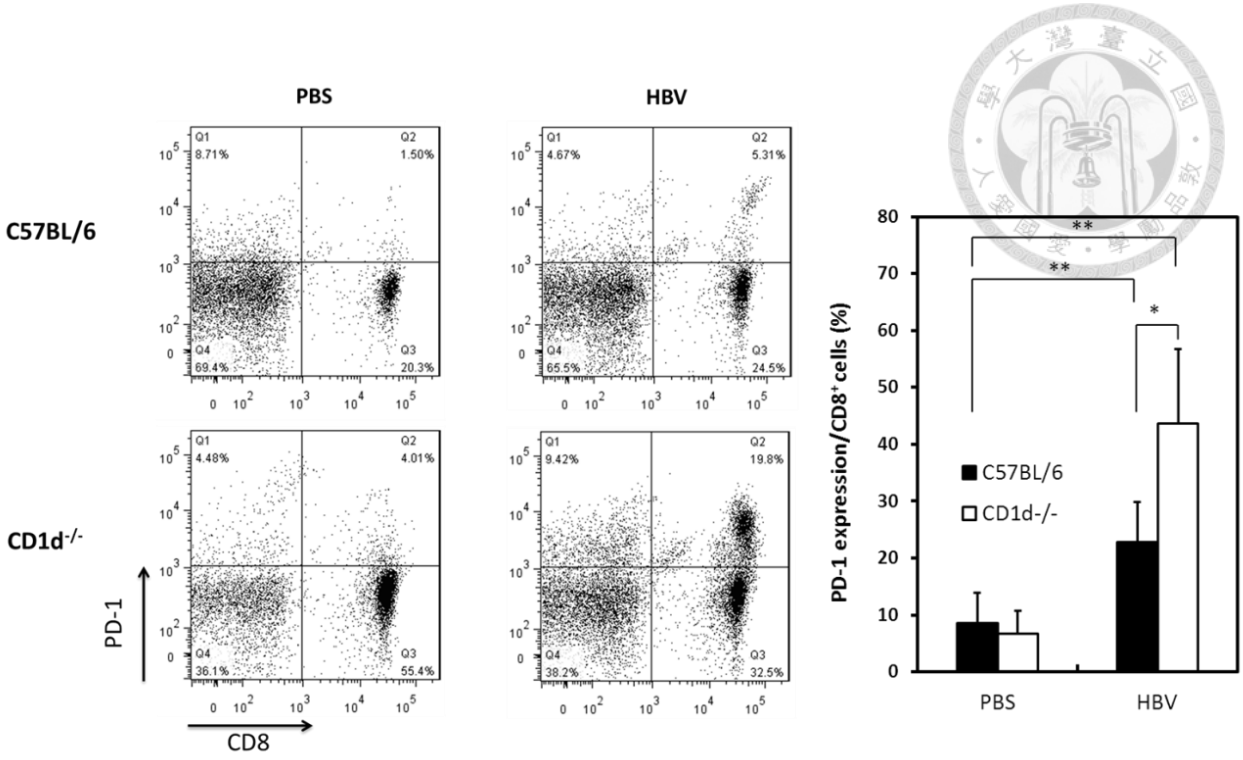


Figure 7 Increased programmed death (PD)-1-expressing $CD8^+$ T cells in liver-infiltrating lymphocytes from $CD1d^{-/-}$ mice compared to C57BL/6 mice after hydrodynamic injection of HBV plasmid

Wild type C57BL/6 and CD1d knockout mice were injected hydrodynamically with HBV plasmid or PBS. Ten weeks post-injection, intrahepatic leukocytes were isolated and PD-1 expression were analyzed by flow cytometry. Error bars represent the SD. *P<0.05, **P<0.01 and ***P<0.001

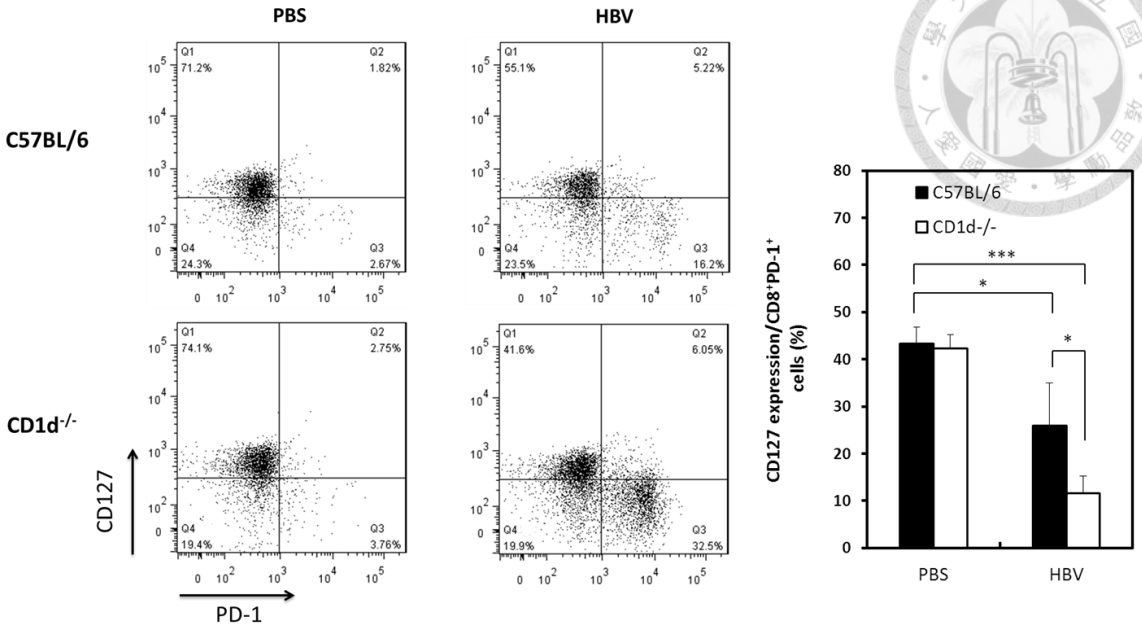


Figure 8 Liver-infiltrating $CD8^+$ lymphocytes in $CD1d^{-/-}$ mice displayed the $PD\text{-}1^{hi}CD127^{low}$-exhausted phenotype after hydrodynamic injection of HBV plasmid

Wild type C57BL/6 and CD1d knockout mice were injected hydrodynamically with HBV plasmid or PBS. Ten weeks post-injection, intrahepatic leukocytes were isolated and PD-1 and CD127 expression by $CD8^+$ T cells were analyzed by flow cytometry. Error bars represent the SD. *P<0.05, **P<0.01 and ***P<0.001

References

1. Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nature reviews Immunology* 2005, **5**(3)**:** 215-229.

2. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP*, et al.* Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. *The Journal of infectious diseases* 1985, **151**(4)**:** 599-603.

3. Chu CM. Natural history of chronic hepatitis B virus infection in adults with emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. *Journal of gastroenterology and hepatology* 2000, **15 Suppl:** E25-30.

4. Yuen MF, Lai CL. Natural history of chronic hepatitis B virus infection. *Journal of gastroenterology and hepatology* 2000, **15 Suppl:** E20-24.

5. Crispe IN. The Liver as a Lymphoid Organ. *Annu Rev Immunol* 2009, **27:** 147-163.

6. Crispe IN. Hepatic T cells and liver tolerance. *Nature reviews Immunology* 2003, **3**(1)**:** 51-62.

7. Racanelli V, Rehermann B. The liver as an immunological organ. *Hepatology* 2006, **43**(2)**:** S54-S62.

8. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A*, et al.* Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* 2012, **18**(7)**:** 1060-1068.

9. Jiang XT, Zhang MX, Lai QT, Huang X, Li YY, Sun J*, et al.* Restored Circulating Invariant NKT Cells Are Associated with Viral Control in Patients with Chronic Hepatitis B. *PloS one* 2011, **6**(12).

10. Fisicaro P, Valdatta C, Boni C, Massari M, Mori C, Zerbini A*, et al.* Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. *Gut* 2009, **58**(7)**:** 974-982.

11. Chen Y, Wei H, Gao B, Hu Z, Zheng S, Tian Z. Activation and function of hepatic NK cells in hepatitis B infection: an underinvestigated innate immune response. *Journal of viral hepatitis* 2005, **12**(1)**:** 38-45.

12. Yang PL, Althage A, Chung J, Maier H, Wieland S, Isogawa M*, et al.* Immune effectors required for hepatitis B virus clearance. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(2)**:** 798-802.

13. Garcia-Rodriguez MJ, Canales MA, Hernandez-Maraver D, Hernandez-Navarro F. Late reactivation of resolved hepatitis B virus infection: an increasing complication post rituximab-based regimens treatment? *American journal of hematology* 2008, **83**(8)**:** 673-675.

14. Mizukoshi E, Sidney J, Livingston B, Ghany M, Hoofnagle JH, Sette A*, et al.* Cellular immune responses to the hepatitis B virus polymerase. *Journal of immunology* 2004, **173**(9)**:** 5863-5871.

15. Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M*, et al.* The Cytotoxic T-Lymphocyte Response to Multiple Hepatitis-B Virus Polymerase Epitopes during and after Acute Viral-Hepatitis. *Journal of Experimental Medicine* 1995, **181**(3)**:** 1047-1058.

16. Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH*, et al.* CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *Journal of virology* 2003, **77**(1)**:** 68-76.

17. Lopes AR, Kellam P, Das A, Dunn C, Kwan A, Turner J*, et al.* Bim-mediated deletion of anti gen-specific CD8(+) T cells in patients unable to control HBV infection. *Journal of Clinical Investigation* 2008, **118**(5)**:** 1835-1845.

18. Benseler V, Warren A, Vo M, Holz LE, Tay SS, Le Couteur DG*, et al.* Hepatocyte entry leads to degradation of autoreactive CD8 T cells. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**(40)**:** 16735-16740.

19. Bowen DG, Zen M, Holz L, Davis T, McCaughan GW, Bertolino P. The site of primary T cell activation is a determinant of the balance between intrahepatic

tolerance and immunity. *Journal of Clinical Investigation* 2004, **114**(5)**:** 701-712.

20. Schurich A, Khanna P, Lopes AR, Han KJ, Peppa D, Micco L*, et al.* Role of the Coinhibitory Receptor Cytotoxic T Lymphocyte Antigen-4 on Apoptosis-Prone CD8 T Cells in Persistent Hepatitis B Virus Infection. *Hepatology* 2011, **53**(5)**:** 1494-1503.

21. Tinoco R, Alcalde V, Yang YT, Sauer K, Zuniga EI. Cell-Intrinsic Transforming Growth Factor-beta Signaling Mediates Virus-Specific CD8(+) T Cell Deletion and Viral Persistence In Vivo. *Immunity* 2009, **31**(1)**:** 145-157.

22. Wherry EJ. T cell exhaustion. *Nature immunology* 2011, **12**(6)**:** 492-499.

23. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH*, et al.* Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006, **439**(7077)**:** 682-687.

24. Shin H, Blackburn SD, Blattman JN, Wherry EJ. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *The Journal of experimental medicine* 2007, **204**(4)**:** 941-949.

25. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A*, et al.* Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nature immunology* 2009, **10**(1)**:** 29-37.

26. Fisicaro P, Valdatta C, Massari M, Loggi E, Biasini E, Sacchelli L*, et al.* Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. *Gastroenterology* 2010, **138**(2)**:** 682-693, 693 e681-684.

27. Tzeng HT, Tsai HF, Liao HJ, Lin YJ, Chen L, Chen PJ*, et al.* PD-1 blockage reverses immune dysfunction and hepatitis B viral persistence in a mouse animal model. *PloS one* 2012, **7**(6)**:** e39179.

28. Zhang Z, Zhang JY, Wherry EJ, Jin B, Xu B, Zou ZS*, et al.* Dynamic programmed death 1 expression by virus-specific CD8 T cells correlates with the outcome of acute hepatitis B. *Gastroenterology* 2008, **134**(7)**:** 1938-1949, 1949

e1931-1933.

29. Kassel R, Cruise MW, Iezzoni JC, Taylor NA, Pruett TL, Hahn YS. Chronically inflamed livers up-regulate expression of inhibitory B7 family members. *Hepatology* 2009, **50**(5)**:** 1625-1637.

30. Webster GJ, Bertoletti A. Control or persistence of hepatitis B virus: the critical role of initial host-virus interactions. *Immunology and cell biology* 2002, **80**(1)**:** 101-105.

31. Micco L, Peppa D, Loggi E, Schurich A, Jefferson L, Cursaro C*, et al.* Differential boosting of innate and adaptive antiviral responses during pegylated-interferon-alpha therapy of chronic hepatitis B. *Journal of hepatology* 2013, **58**(2)**:** 225-233.

32. Bertoletti A, Gehring AJ. The immune response during hepatitis B virus infection. *The Journal of general virology* 2006, **87**(Pt 6)**:** 1439-1449.

33. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. *Science* 1999, **284**(5415)**:** 825-829.

34. Sato S, Li K, Kameyama T, Hayashi T, Ishida Y, Murakami S*, et al.* The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. *Immunity* 2015, **42**(1)**:** 123-132.

35. Huang LR, Wu HL, Chen PJ, Chen DS. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(47)**:** 17862-17867.

36. Xu L, Yin WW, Sun R, Wei HM, Tian ZG. Liver type I regulatory T cells suppress germinal center formation in HBV-tolerant mice. *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**(42)**:** 16993-16998.

37. Tzeng HT, Tsai HF, Chyuan IT, Liao HJ, Chen CJ, Chen PJ*, et al.* Tumor necrosis factor-alpha induced by hepatitis B virus core mediating the immune response

for hepatitis B viral clearance in mice model. *PloS one* 2014, **9**(7)**:** e103008.

38. Xu L, Yin WW, Sun R, Wei HM, Tian ZG. Kupffer Cell-Derived IL-10 Plays a Key Role in Maintaining Humoral Immune Tolerance in Hepatitis B Virus-Persistent Mice. *Hepatology* 2014, **59**(2)**:** 443-452.

39. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 2004, **22:** 817-890.

40. Mori L, De Libero G. T cells specific for lipid antigens. *Immunol Res* 2012, **53**(1-3)**:** 191-199.

41. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* 2007, **25:** 297-336.

42. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K*, et al.* CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997, **278**(5343)**:** 1626-1629.

43. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. Opinion - NKT cells: what's in a name? *Nature Reviews Immunology* 2004, **4**(3)**:** 231-237.

44. Rhost S, Sedimbi S, Kadri N, Cardell SL. Immunomodulatory Type II Natural Killer T Lymphocytes in Health and Disease. *Scand J Immunol* 2012, **76**(3)**:** 246-255.

45. Guy CS, Mulrooney-Cousins PM, Churchill ND, Michalak TI. Intrahepatic expression of genes affiliated with innate and adaptive immune responses immediately after invasion and during acute infection with woodchuck hepadnavirus. *Journal of virology* 2008, **82**(17)**:** 8579-8591.

46. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* 1997, **6**(4)**:** 459-467.

47. Boettler T, Panther E, Bengsch B, Nazarova N, Spangenberg HC, Blum HE*, et al.* Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells

during acute resolving hepatitis B virus infection. *Journal of virology* 2006, **80**(7)**:** 3532-3540.

48. Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T*, et al.* Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *Journal of virology* 2007, **81**(8)**:** 4215-4225.

49. Bramson JL, Graham FL, Gauldie J. The use of adenoviral vectors for gene therapy and gene transfer in vivo. *Current opinion in biotechnology* 1995, **6**(5)**:** 590-595.

50. Shayakhmetov DM, Gaggar A, Ni S, Li ZY, Lieber A. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *Journal of virology* 2005, **79**(12)**:** 7478-7491.

51. Liu Q, Zaiss AK, Colarusso P, Patel K, Haljan G, Wickham TJ*, et al.* The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors. *Human gene therapy* 2003, **14**(7)**:** 627-643.

52. Hartman ZC, Kiang A, Everett RS, Serra D, Yang XY, Clay TM*, et al.* Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo. *Journal of virology* 2007, **81**(4)**:** 1796-1812.

53. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B*, et al.* Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Molecular therapy : the journal of the American Society of Gene Therapy* 2001, **3**(5 Pt 1)**:** 697-707.

54. Zhu J, Huang X, Yang Y. Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and -independent pathways. *Journal of virology* 2007, **81**(7)**:** 3170-3180.

55. Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* 1996, **2**(10)**:** 1104-1108.

56. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y*, et al.* Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *Journal of immunology* 1999, **163**(9)**:** 4647-4650.

57. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *The Journal of clinical investigation* 2004, **114**(10)**:** 1379-1388.

58. Kitamura H, Ohta A, Sekimoto M, Sato M, Iwakabe K, Nakui M*, et al.* alpha-galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cellular immunology* 2000, **199**(1)**:** 37-42.

59. Ishikawa H, Tanaka K, Kutsukake E, Fukui T, Sasaki H, Hata A*, et al.* IFN-gamma production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8+ T cells. *Virology* 2010, **407**(2)**:** 325-332.

60. van Dommelen SL, Tabarias HA, Smyth MJ, Degli-Esposti MA. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. *Journal of virology* 2003, **77**(3)**:** 1877-1884.

61. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nature reviews Microbiology* 2007, **5**(6)**:** 405-417.

62. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I*, et al.* Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 1997, **278**(5343)**:** 1623-1626.

63. Bedel R, Matsuda JL, Brigl M, White J, Kappler J, Marrack P*, et al.* Lower TCR repertoire diversity in Traj18-deficient mice. *Nature immunology* 2012, **13**(8)**:** 705-706.

64. Chandra S, Zhao M, Budelsky A, de Mingo Pulido A, Day J, Fu Z*, et al.* A new mouse strain for the analysis of invariant NKT cell function. *Nature immunology* 2015, **16**(8)**:** 799-800.