Liver-specific HBsAg transgenic mice are over-sensitive to Poly(I:C)-induced liver injury in NK cell- and IFN-γ-dependent manner ☆

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Background/Aims: The role of natural killer (NK) cells in the development of hepatitis B virus (HBV)-associated liver injury remains obscure. In this study, we elucidated the role of NK cells in liver injury of HBsAg transgenic mice (HBs-B6), a mimic of human healthy chronic HBsAg carriers, triggered by polyinosinic:polycytidylic acid [Poly(I:C)].

Methods: HBs-B6 or wild B6 mice were intraperitoneally injected with Poly(I:C) at different doses. Liver injury was evaluated by serum transaminase activity and histopathologic changes.

Results: HBs-B6 mice were over-sensitive to Poly(I:C)-induced liver injury, which was absolutely dependent on the presence of NK cells and IFN-γ produced by intrahepatic NK cells. Much stronger IFN-γ receptor expression was observed on hepatocytes of HBs-B6 mice, which was significantly enhanced by Poly(I:C) injection. Treatment with IFN-γ *in vitro* triggered much higher activation of downstream signals (pSTAT1-IRF-1) in hepatocytes of HBs-B6 mice. Depletion of Kupffer cells and neutralization of endogenous IL-12 did not affect Poly(I:C)-induced over-sensitive liver injury in HBs-B6 mice.

Conclusions: NK cells played a critical role in an IFN-γ dependent, Kupffer cell- and IL-12-independent manner in over-sensitive liver injury triggered by Poly(I:C) in murine chronic HBsAg carriers.

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Keywords: Hepatitis B virus; Liver injury; Natural killer cell; IFN-γ; Polyinosinic:polycytidylic acid

1. Introduction

Hepatitis B virus (HBV) primarily infects hepatocytes and causes a series of liver diseases such as acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, which are among the most important human health problems worldwide [1–3]. It has been reported that the immune responses are fundamental for viral clearance and pathogenesis during HBV infection; however, the precise mechanisms of innate immune responses

responsible for the autoimmune-associated liver injury are poorly defined [3]. Liver is a lymphoid organ with an overwhelming innate immune system [4–7]. Natural killer (NK) cells are abundant in the normal liver, accounting for around one-third of intrahepatic lymphocytes [4,5]. Available evidence has indicated that hepatic NK cells play an important anti-viral role during HBV infection, but the role in the development of HBV-associated liver injury remains obscure [8].

Due to the limited host range of HBV and difficulties in getting human liver samples, autoimmune liver injury models and HBV transgenic mouse models were applied to investigate the role of NK cells in liver injury. Activated hepatic NK cells are able to kill hepatocytes and induce liver injury such as viral liver infection (adenovirus), Pseudomonas aeruginosa exotoxin A (PEA) injection and carrageenan administration [9–11], and polyinosinic:polycytidylic acid [Poly(I:C)] injection as reported by Makoto Ochi et al. and us [12,13]. Mole-

cules including IFN-γ, IL-12, Perforin and TRAIL contributed to NK cell cytotoxicity against hepatocytes [9,11–13]. Furthermore, pre-activated T cells by a low dose of ConA could enhance the ability of Poly(I:C)-activated NK cells to cause more severe hepatocyte injury [14]. However, activated NK cells in some cases are not involved in liver injury, as reported in ConA-induced hepatitis or α-GalCer-induced liver injury [15–17]. In HBV transgenic mice, activated NK cells caused liver injury when inhibiting HBV replication [18–22]. However, Guidotti et al. demonstrated that activated NK cells contributed to viral clearance without any cytopathic effects on liver in the HBV-infected chimpanzee [23]. So the precise role of NK cells in the liver injury during HBV infection deserves further investigation.

It is extensively recognized that ten percent of adults and 90% of children become persistent virus carriers after HBV infection. In this study, hepatitis B surface antigen (HBsAg) transgenic mice, which mimic human healthy chronic HBsAg carriers [24,25], were triggered by a synthetic dsRNA analogue Poly(I:C), a ligand of toll-like receptor 3 (TLR3) and also a well-known potent activator for NK cells [13,14,26]. In our previous study, injection of Poly(I:C) could preferentially recruit and activate NK cells in liver and induce mild liver injury in wild mice [13,14]. Here, we further demonstrated that the HBsAg transgenic mice were over-sensitive to Poly(I:C)-induced liver injury, which was absolutely dependent on hepatic NK cells. NK cells played their critical role in an IFN-γ dependent and Kupffer cell- and IL-12-independent manner in these HBsAg transgenic mice, which was completely different from wild mice as we previously observed [13].

2. Materials and methods

2.1. Mice

HBsAg transgenic mice C57BL/6J-TgN (AlblHBV) 44Bri (named as HBs-B6 mice in this study) were used, which express HBsAg in serum, liver and kidney tissues to mimic human healthy chronic HBsAg carriers [24,25]. Eight- to 10-week-old male HBs-B6 mice were obtained from VITALRIVER Experiment Animal Company (Beijing) which purchased them from Jackson Lab and bred them for us. Their littermates C57BL/6J mice (B6 mice) were also obtained as control. All mice were maintained under specific-pathogen-free conditions with human care. Experimental procedures were conducted in accordance with experimental animal's guidelines.

2.2. Reagents

Poly(I:C) (Sigma Chemical Co., St Louis, MO) was dissolved in the pyrogen-free phosphate-buffered saline (PBS) at the concentration of 1 mg/ml and intraperitoneally injected to mice at indicated doses. The mAbs for flow cytometry analysis included FITC-conjugated anti-NK1.1, PE-conjugated anti-IFN-γ, PE-CY5-conjugated anti-CD3e, Biotin-anti-CD119 (IFN-γ R α chain) (BD PharMingen, San Diego), FITC-conjugated streptavidin (eBioscience, San Diego). Recombinant mIFN-γ (Pepro Tech INC) was dissolved in pyrogen-

free PBS at the concentration of 25 μg/ml, and was intravenously injected to mice at a series of doses to observe its effect on liver injury. For in vitro experiments, cultured hepatocytes were treated with recombinant mIFN-γ (2 ng/ml) or PBS. Anti-phospho-STAT1 (Tyr701) and anti-IRF-1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody was obtained from Boster (Wuhan, China). Horseradish peroxidase-conjugated secondary Abs was obtained from Promega (Beijing, China).

2.3. Assay for serum transaminase activity

Serum samples from individual mice were obtained at indicated time points. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using commercial available kit (Rong Sheng, Shanghai, China) and calculated from the standard curve.

2.4. H–E staining

Liver tissues were fixed in 10% neutral buffered formalin at least for 24 h and then embedded in paraffin. Five-micron sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to determine morphologic changes.

2.5. Cell depletion

A dose of 50 μl anti-ASGM1 antibody with the concentration of 1 mg/ml (Wako Pure Chemical Industries Ltd., Osaka, Japan) or same amount of control rabbit IgG (Sino-American Biotechnology Co., Beijing, China) was intravenously injected into mice 24 h before the following treatment to deplete NK cells [14]. To deplete Kupffer cells in vivo, gadolinium chloride (GdCl₃) (Sigma Chemical Co., St. Louis, MO) was intravenously injected at 10 mg/kg body weight 24 h before the following treatment [27,28]. These protocols resulted in a ⩾90% decrease in the number of the indicated cells, which was confirmed by flow cytometry.

2.6. Cytokine neutralization

Anti-IFN-γ neutralizing mAb (R4-6A2, IgG₁) and anti-interleukin (IL)-12 neutralizing mAb (anti-IL-12 p40, R2-10F6, IgG₂b) were partially purified from hybridoma culture supernatant by ammonium sulfate precipitation obtained from American Type Culture Collection (Manassas, VA, USA). Mice were given three injections of the indicated mAb (50 μg per mouse) intraperitoneally on three consecutive days before the following treatment to neutralize the cytokine in vivo. Control rat IgG (Sino-American Biotechnology Co.) was injected at equivalent doses and schedules.

2.7. Isolation and culture of primary mouse hepatocytes

The isolation and culture of primary mouse hepatocytes were performed as described [29]. Followed by treatment with IFN-γ for various time periods, downstream signals of hepatocytes were determined by Western blotting.

2.8. Flow cytometry analysis

Hepatic mononuclear cells (MNCs) were prepared as described previously [24]. For the intracellular cytokine assay, MNCs were cultured in the presence of 30 ng/ml PMA, 1 μg/ml ionomycin (Calbiochem, Darmstadt, Germany) and 6 μM monensin (Sigma Chemical Co., St. Louis, MO) for 4 h in humidified 5% CO₂ at 37 °C. After blocking with anti-FcR, MNCs were stained in darkness at 4 °C for 30 min with the indicated mAbs FITC-conjugated anti-NK1.1 and PE-CY5-conjugated anti-CD3e for the surface antigens. Subsequently, cells were fixed and permeabilized using 100 μl of cytofix and cytoperm solution (eBioscience, San Diego, CA, USA), respectively, and then

stained with PE-conjugated anti-IFN-γ mAb. For IFN-γ receptor assay, after blocking with anti-FcR isolated hepatocytes were stained with Biotin-anti-CD119 in darkness at 4 °C for 30 min, and then washed three times before being stained with FITC-conjugated streptavidin in darkness at 4 °C for 30 min. Stained cells were washed three times and then examined by FACScalibur (Becton–Dickinson) and analyzed with WinMDI 2.8 software.

2.9. Western blotting

Cells were lysed in cell lysis buffer for western and IP (Beyotime, China) added with 1 mM PMSF for 30 min in ice, and then centrifuged at 12,000g for 15 min at 4 °C. The supernatants were mixed in Laemmli loading buffer, boiled for 5 min, and then subjected to SDS–PAGE. After electrophoresis, proteins were transferred onto PVDF membranes (Millipore Corporation, Billerica, USA), and blotted against primary Abs overnight at 4 °C. Membranes were washed with 0.1% (vol/vol) Tween 20 in TBS (pH 7.6) and incubated with a 1:2500 dilution of horseradish peroxidase-conjugated secondary Abs for 60 min at room temperature. Protein bands were visualized by ECL reaction (Pierce).

2.10. Statistical analysis

Differences between the groups were analyzed by Student's *t* test and *P* < 0.05 was considered statistically significant.

3. Results

3.1. HBs-B6 mice were over-sensitive to Poly(I:C)-induced liver injury

As previously reported by us [13], Poly(I:C)-induced mild liver injury in wild B6 mice at a high dose of 20 μg/g body weight. However, a lower dose of Poly(I:C) (7.5 μg/g body weight) could induce remarkable elevation of ALT in HBs-B6 mice. As shown in Fig. 1, Poly(I:C) injection induced much higher elevations of serum ALT and AST in HBs-B6 mice than wild B6 mice, even with a much low dose (3 μg/g body weight). Furthermore, there was no significant difference in the peak level of serum ALT between a low dose of Poly(I:C) (3 μg/g body weight) – and a high dose of Poly(I:C) (30 μg/g body weight) – treated HBs-B6 mice. In wild B6 mice, there were only slight elevations of serum ALT and AST after 30 μg/g body weight Poly(I:C) injection. Liver histology further confirmed that injection of low dose of Poly(I:C) (3 μg/g body weight) induced severe hepatocyte necrosis and inflammation in HBs-B6 mice, but no morphologic changes in the liver of wild B6 mice (Fig. 2). These results demonstrated that HBs-B6 mice were over-sensitive to Poly(I:C)-induced liver injury.

3.2. NK cells played a critical role in the over-sensitive liver injury in HBs-B6 mice after Poly(I:C) injection

NK cells were preferentially accumulated and activated in the liver after Poly(I:C) injection both in wild B6 mice and HBs-B6 mice [13,24]. We previously
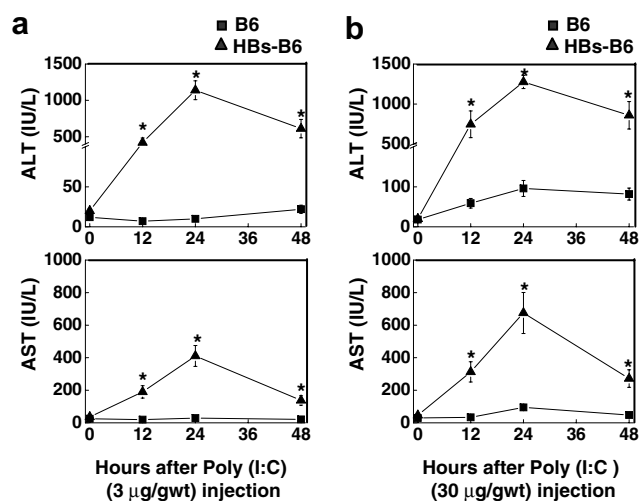

Fig. 1. Serum ALT/AST in HBs-B6 mice after Poly(I:C) injection. Poly(I:C) was injected intraperitoneally to HBs-B6 mice and wild B6 mice with different doses. Serum ALT and AST levels were measured at 0, 12, 24 and 48 h after Poly(I:C) injection. (a) Levels of serum ALT/AST after 3 μg/g body weight Poly(I:C) injection. (b) Levels of serum ALT/AST after 30 μg/g body weight Poly(I:C) injection. Values are shown as means ± SEM from five mice at each time point in each group. *P < 0.05 vs. B6 mice.

reported that Poly(I:C)-activated NK cells were implicated in the mild liver injury in wild B6 mice [13]. In order to directly elucidate the role of NK cells in the over-sensitive liver injury induced by low dose of Poly(I:C) in HBs-B6 mice, depletion of NK cells was achieved by pretreatment with anti-ASGM1 antibody. As shown in Fig. 3, depletion of NK cells significantly decreased levels of serum ALT and AST by effectively
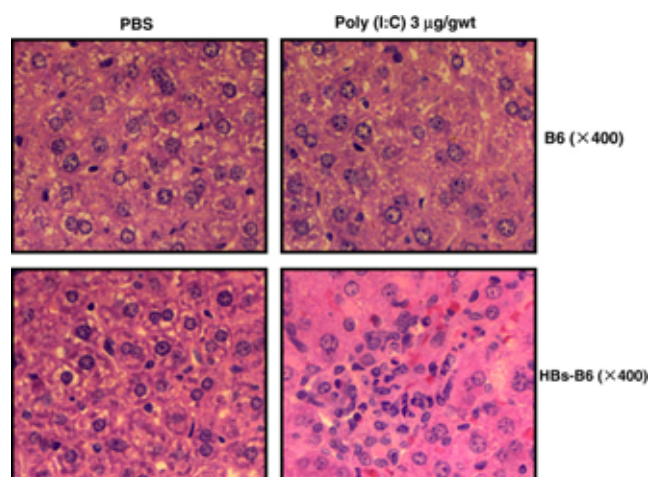

Fig. 2. Severe hepatocyte necrosis and inflammation in HBs-B6 mice after low dose of Poly(I:C) injection. Twenty-four hours after 3 μg/g body weight Poly(I:C) or PBS injection, liver samples were collected from HBs-B6 and wild B6 mice, and then liver sections were prepared and stained with hematoxylin and eosin (original magnification 400×). These were from a single experiment representative of three independent experiments. [This figure appears in colour on the web.]

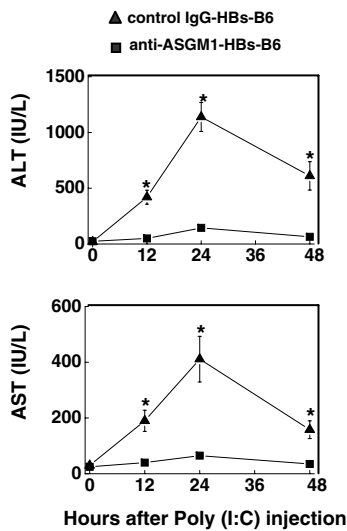　　　　　　　　　　　　　　*Y. Chen et al. / Journal of Hepatology 47 (2007) 183–190*

Fig. 3. Depletion of NK cells attenuated liver injury induced by low dose of Poly(I:C) in HBs-B6 mice. HBs-B6 mice were treated with control rabbit IgG or anti-ASGM1 antibody for 24 h, followed by injection of Poly(I:C) (3 µg/g body weight). At 0, 12, 24 and 48 h after Poly(I:C) injection, serum ALT and AST levels were measured. Values are shown as means ± SEM from four mice at each time point in each group. $^{*}P < 0.05$ vs. corresponding control group.

abrogating low dose of Poly(I:C)-induced liver injury in HBs-B6 mice, suggesting that the over-sensitivity to Poly(I:C)-induced liver injury of HBs-B6 mice was dependent on NK cells.

3.3. NK cell-mediated liver injury was dependent on IFN-γ in HBs-B6 mice after Poly(I:C) injection

After low dose of Poly(I:C) injection, largely enhanced IFN-γ production by intrahepatic NK cells was observed in both HBs-B6 mice and wild B6 mice (Fig. 4a). However, neutralization of endogenous IFN-γ by pretreatment with anti-IFN-γ mAb absolutely alleviated the liver injury induced by low dose of Poly(I:C) in HBs-B6 mice (Fig. 4b), indicating that IFN-γ played a crucial role in over-sensitive liver injury of HBs-B6 mice. Further, HBs-B6 or wild B6 mice were injected with different doses of recombinant mIFN-γ, and the results demonstrated that a dose of 7.5 µg recombinant mIFN-γ could induce marked liver injury in HBs-B6 mice, but not in wild B6 mice (Fig. 4c), indicating that HBs-B6 mice were hypersensitive to IFN-γ in liver injury.

As shown in Fig. 5a, the expression of IFN-γ receptor on hepatocytes of HBs-B6 mice was much stronger than that of wild B6 mice (12.89% vs. 1.00%); Poly(I:C) injection significantly enhanced the expression of IFN-γ receptor on hepatocytes of HBs-B6 mice, but not on hepatocytes of wild B6 mice. Further, the higher activation of downstream signals pSTAT1-IRF-1 in hepatocytes of HBs-B6 mice after treatment with IFN-γ *in vitro* was observed (Fig. 5b).
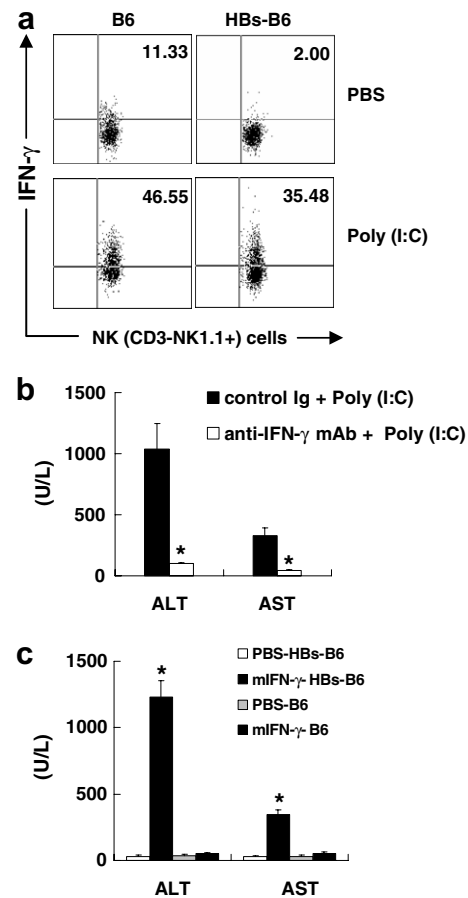

Fig. 4. NK cell-mediated liver injury was dependent on IFN-γ in HBs-B6 mice after low dose of Poly(I:C) injection. (a) Twenty-four hours after Poly(I:C) (3 µg/g body weight) or PBS injection, liver MNCs were prepared and then analyzed by flow cytometry for intracellular IFN-γ of NK cells. A representative example of three independent experiments is shown. (b) HBs-B6 mice were treated with anti-IFN-γ mAb to neutralize endogenous IFN-γ before Poly(I:C) (3 µg/g body weight) injection. Serum ALT and AST were determined 24 h after Poly(I:C) injection. Data are shown as means ± SEM from four mice in each group. $^{*}P < 0.05$ as comparison with the control. (c) A dose of 7.5 µg recombinant mIFN-γ or PBS was injected iv. to HBs-B6 and wild B6 mice. At 24 h after IFN-γ injection, serum ALT and AST levels were determined. Data are shown as means ± SEM from four mice in each group. $^{*}P < 0.05$ vs. corresponding control group.

3.4. NK cell-mediated liver injury was Kupffer cell- and IL-12-independent in HBs-B6 mice after Poly(I:C) injection

We previously reported that Kupffer cell-produced IL-12 played critical roles in Poly(I:C)-induced NK cell accumulation and the subsequent mild liver injury in wild B6 mice [13]. However, as shown by the levels of serum ALT and AST in Fig. 6, depletion of Kupffer cells by pretreatment of GdCl₃ did not change the Poly(I:C)-induced liver injury in HBs-B6 mice; and neutralization of endogenous IL-12 by pretreatment with anti-IL-12 mAb neither affected the liver injury. These results
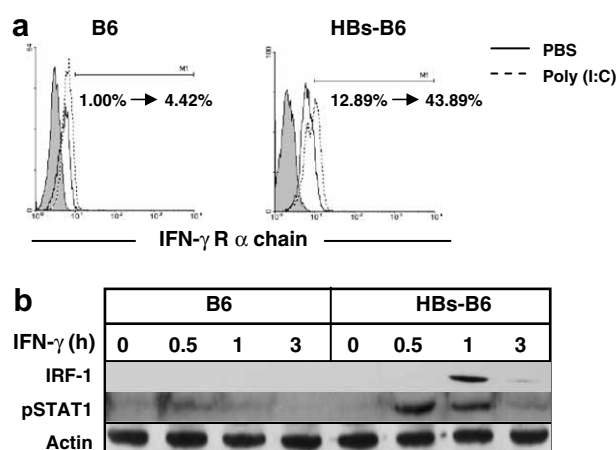
Fig. 5. Stronger expression of IFN-γ receptor and higher IFN-γ-activated downstream signals pSTAT-1-IRF-1 in hepatocytes of HBs-B6 mice. (a) Six hours after Poly(I:C) (3 μg/g body weight) or PBS injection, hepatocytes of HBs-B6 and wild B6 mice were prepared and then analyzed for IFN-γ R α chain by flow cytometry. A representative example of three independent experiments is shown. The gray histogram is for the negative control in each group. (b) Western blotting analyses of pSTAT-1 and IRF-1 from primary HBs-B6 and wild B6 mouse hepatocytes treated with IFN-γ (2 ng/ml) *in vitro* for 0, 0.5, 1 and 3 h, respectively.

indicated that Kupffer cells and IL-12 were not necessary for NK cells to mediate the Poly(I:C)-triggered liver injury in HBs-B6 mice.
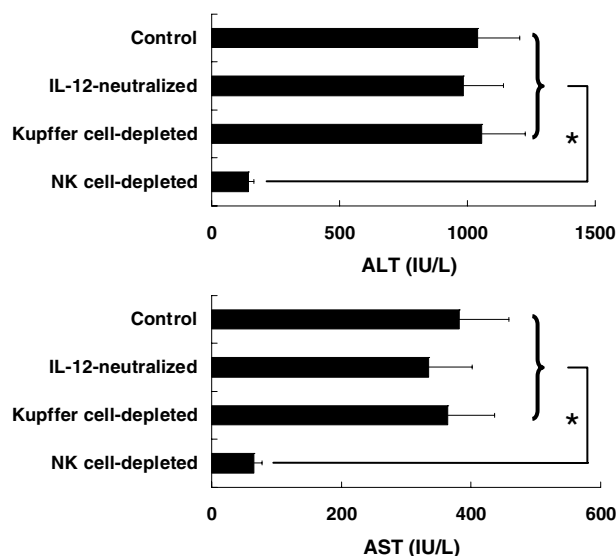

Fig. 6. NK cell mediated-liver injury was Kupffer cell- and IL-12-independent in HBs-B6 mice after low dose of Poly(I:C) injection. Depletion of NK cells was achieved by pretreatment with anti-ASGM1 antibody and depletion of Kupffer cells was achieved by pretreatment with GdCl₃ 24 h before Poly(I:C) injection in HBs-B6 mice. Endogenous IL-12 was neutralized by pretreatment with anti-IL-12 mAb on three consecutive days before Poly(I:C) injection in HBs-B6 mice. Serum ALT and AST were determined 24 h after Poly(I:C) (3 μg/g body weight) injection. Values are shown as means ± SEM from four mice in each group. $^*P < 0.05$ vs. Kupffer cell-depleted or IL-12-neutralized or control.

4. Discussion

In this study, we demonstrate that Poly(I:C) triggers over-sensitive liver injury of murine chronic HBsAg carriers in an NK cell-dependent manner. We have previously reported that Poly(I:C)-induced mild liver injury was dependent on NK cells, and Kupffer cells played critical roles in Poly(I:C)-induced NK cell accumulation and the subsequent liver injury in wild mice [13]. However, in current HBsAg transgenic mice Poly(I:C)-induced liver injury is independent of Kupffer cells (Fig. 6). After Poly(I:C) injection, more NK cells were accumulated in the liver and persisted longer in HBsAg transgenic mice [24], of which the actual molecular mechanisms remained unclear. So, it seems that mechanisms of NK cell activation by Poly(I:C) are different in Kupffer cell-dependence in HBsAg transgenic mice and wild mice. In addition, we reported that NKT cells were not involved in Poly(I:C)-induced NK cell-mediated liver injury [13], which was further confirmed by us in HBsAg transgenic mice by comparison of NKT/NK cell depletion by anti-NK1.1 mAb with NK cell depletion by anti-ASGM-1 antibody since lack of HBsAg⁺ CD1d⁻/⁻ mice (data not shown), indicating that the over-sensitive liver injury was mainly dependent on NK cells. Interestingly, NK cells did not contribute to α-GalCer-induced NKT cell-mediated hepatocyte injury in wild mice [17], but injection of α-GalCer into HBsAg transgenic mice induced more severe NKT cell-mediated liver injury, which was dependent on NK cells [30]. So, taken together, NK cells play the critical role in over-sensitive liver injury of HBsAg transgenic mice after triggering with Poly(I:C) or α-GalCer although the dependence on NKT cells is absolutely different.

As a self-antigen, HBsAg could not usually induce specific immune responses to HBsAg in the immunologically tolerant transgenic mice. As previously reported [31–33], structure of virus such as mouse cytomegalovirus (MCMV) m157 may be recognized as the ligand of activating or inhibitory NK cell receptor (so called "none-self"), by which NK cells may attack self-tissue. Until now, no report including our preliminary study (data not shown) demonstrates that HBsAg has the character. Also, virus proteins such as MCMV m155-, m145-, m152- or m138-encoded glycoprotein may induce the virus infected cells to down-express or up-express the ligands of activating NK cell receptor (so called "induced-self" or "missing-self", respectively), by which NK cells may attack self-tissue too [34–38]. We practiced the experiments to explore the ligands of activating NK cell receptor on hepatocytes of HBsAg transgenic mice, and no significant change was found (data not shown). On the other hand, after Poly(I:C) injection, NK cells were preferentially accumulated and activated in the liver [13,24] and produced large amount of IFN-γ similarly (Fig. 4a) in both HBsAg

transgenic mice and wild B6 mice. As previously reported by us [13], IFN-γ were not responsible for Poly(I:C)-induced mild liver injury in wild mice, although IFN-γ has been considered to play a critical role in liver injury induced by other immunostimulants [39–41]. However, in the present study, IFN-γ was involved in Poly(I:C)-induced over-sensitive liver injury in HBsAg transgenic mice after verifying by IFN-γ neutralization and rmIFN-γ injection (Fig. 4). Gilles et al. reported that HBsAg-positive hepatocytes are extraordinarily sensitive to the cytolytic effect of IFN-γ *in vivo* [42], but the IFN-γ-producing cells as well as effector cells were not delineated in their study. Here, we demonstrated that NK cells were the effectors to mediate the over-sensitive liver injury in an IFN-γ-dependent manner in HBsAg transgenic mice. As demonstrated in ConA-induced hepatitis or LPS/D-galactosamine-induced liver damage, IFN-γ plays an important role in the development of hepatic inflammation and liver injury through induction of chemokines, adhesive molecules, and proapoptotic proteins by activation of STAT1 [29,43–46]. We observed higher expressions of IFN-γ receptor on hepatocytes of HBsAg transgenic mice and stronger IFN-γ signaling of pSTAT1-IRF-1, which might account for the hypersensitivity to IFN-γ-induced hepatocyte injury in HBsAg transgenic mice (Fig. 5).

TNF-α is another important inflammatory cytokine in liver injury [41,47,48], but does not contribute to Poly(I:C)-induced liver injury in wild mice [13]. In HBsAg transgenic mice, HBsAg-positive hepatocytes were not hypersensitive to the cytolytic effect of TNF-α [42]. Since neutralization of IFN-γ completely inhibited the over-sensitive liver injury induced by Poly(I:C) in this study, it was speculated that TNF-α was not necessary to the over-sensitive liver injury in HBsAg transgenic mice. Poly(I:C) can stimulate the production of IL-12, which then promotes NK cell accumulation in the liver [49,50]. In Poly(I:C)-induced liver injury of wild mice, Kupffer cell-derived IL-12 played critical roles in NK cell function [13]. IL-12 has been shown to exacerbate ConA-induced hepatitis in wild mice [51]. However, in this study, the Poly(I:C)-induced over-sensitive liver injury in HBsAg transgenic mice was independent of IL-12. We still do not know why NK cell-derived IFN-γ is strong enough to induce liver injury in HBsAg transgenic mice, regardless of the presence of IL-12 (Fig. 6).

It has been known that human healthy HBsAg carriers are susceptible to hepatocyte injury, as a serious risk for development of liver cirrhosis and hepatocellular carcinoma [52], however, the pathogenesis has not been well understood. During HBV infection, a variety of elements such as stresses, infections, pregnancy and use or withdrawal of immunosuppressive therapies are disadvantageous to induce hepatocyte injury [53]. In the

present study, by using HBsAg transgenic mice that mimic human chronic HBsAg carriers, we used Poly(I:C), as a mimicking exoteric stimulant of virus infection [13,14,26], to investigate the underlying innate immune mechanisms of hepatocyte injury during HBV infection. Our study elucidated for the first time that activated NK cells were responsible for the over-sensitivity to hepatocyte injury of chronic HBsAg carriers in an IFN-γ-dependent manner.

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