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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-

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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 ($GdCl_3$) (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 $\geqslant$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, IgG_1) and anti-interleukin (IL)-12 neutralizing mAb (anti-IL-12 p40, R2-10F6, IgG_2b) 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% CO_2 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

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