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Granzyme H of Cytotoxic Lymphocytes Is Required for Clearance of the Hepatitis B Virus through Cleavage of the Hepatitis B Virus X Protein

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The granule exocytosis pathway of cytotoxic lymphocytes plays critical roles in eradication of intracellular viruses. However, how hepatitis B virus (HBV) clearance has not been defined. To clarify immune mechanisms underlying inhibition of the HBV replication, the relationship between granzyme H (GzmH) and HBV clearance was investigated. In this study, we found that the granule exocytosis pathway can inhibit HBV replication without induction of cytolysis of the infected cells. GzmH is essential for HBV eradication. The HBx protein (HBx), required for the replication of HBV, is cleaved at Met20 by GzmH. GzmH inhibitor can abolish GzmH- and lymphokine-activated killer cell-mediated HBx degradation and HBV clearance. An HBx-deficient HBV is resistant to GzmH- and lymphokine-activated killer cell-mediated viral clearance. Adoptive transfer of GzmH-overexpressing NK cells into HBV carrier mice facilitates in vivo HBV eradication. Importantly, low GzmH expression in cytotoxic lymphocytes of individuals is susceptible to HBV infection and hepatocellular carcinoma. These results indicate that GzmH might be detected as a potential parameter for diagnosis of HBV infection and hepatocellular carcinoma. The Journal of Immunology, 2012, 188: 824–831.

Cytotoxic lymphocytes are major players that eliminate transformed tumor cells or cells infected with intracellular pathogens (1, 2). In this process, pore-forming protein perforin and cytotoxic protease granzymes (Gzms) are released from their granules into the immunological synapse formed between cytotoxic cells and their targets. Gzms are a highly conserved set of serine proteases. After they are delivered into target cells by perforin, they can cleave cellular or viral proteins, leading to the destruction of target cells or inhibition of viral replication (3, 4). Humans express five Gzms (A, B, K, H, and M). Because GzmA and B are the most abundant proteases of the Gzm family in humans and mice, their functions have been extensively investigated (1). Recently, we and other groups have defined some roles of orphan Gzms (H, K, and M) (5–8). However, their precise functions have not been clearly defined yet.

GzmH harbors chymotrypsin-like chymase activity and cleaves their substrates after Tyr, Phe, or Met in the PI site (9, 10). We and Fellows et al. (5, 6) showed that GzmH induces cell death with different death pathways. Interestingly, GzmH can directly cleave two adenoviral proteins essential for viral replication, the adenovirus DNA-binding protein and the adenoavirous 100K assembly protein for virus assembly (3). GzmH can also cleave a multifunctional phosphoprotein La to disrupt hepatitis C virus–internal ribosome entry site-mediated translational activity (11). These findings suggest that GzmH exerts direct antiviral activity through cleaving viral or host proteins.

Hepatitis B virus (HBV) infection leads to acute or chronic hepatitis, which is a major cause of liver diseases worldwide (12). Chronic infection of HBV results in a drastically increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC). The HBV virus is a member of hepadnaviridae family, for which the genome is a relaxed circular, partially dsDNA. The HBV genome contains four open reading frames, encoding the viral envelope protein, the viral core protein, the reverse transcriptase, and the HBV X protein (HBx). The HBx is a 16.5-kDa multifunctional regulator that is essential for virus replication (13, 14). To clarify immune mechanisms underlying inhibition of the HBV replication, the relationship between GzmH and HBV clearance was investigated. In this study, we demonstrate for the first time, to our knowledge, that lymphokine-activated killer (LAK) cells inhibit HBV replication in HepG2.2.15 cells without cytolysis of target cells. GzmH participates in the clearance of HBV virus through cleavage of the HBx protein. An HBx-deficient HBV is resistant to LAK cell- or GzmH-mediated viral clearance. Adoptive transfer of GzmH-expressing NK cells into an HBV mouse model facilitates HBV clearance. Importantly, low GzmH expression in cytotoxic lymphocytes is susceptible to HBV infection in individual persons.

Materials and Methods

Cell lines, Abs, and reagents

HepG2 and HepG2.2.15 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2.2.15 cells were maintained in medium containing 380 μg/ml G418. K562 cells were cultured in RPMI 1640 medium. Commercial Abs were mouse mAbs against HBx (Chemicon International), FLAG (Sigma-Aldrich), CD56-Alexa 488 (eBioscience), CD56 (Goldenbridge, Beijing, China), GzmH [a kind gift from Dr. Joseph Trapani, Peter MacCallum
Cancer Centre, East Melbourne, VIC, Australia (15), rabbit polyclonal Ab against NM23-H1 (Santa Cruz Biotechnology). GzmH (Abcam), Alexa 488-conjugated donkey anti-mouse IgG (Molecular Probes). Alexa 594-conjugated donkey anti-rabbit IgG (Molecular Probes), PE-conjugated goat anti-mouse IgG (eBioscience), and PE-conjugated goat anti-rabbit IgG (eBioscience). Concentracycin A (CMA), streptolysin O (SLO), and Hoechst 33342 were from Sigma-Aldrich.

Plasmid construction

Active and mutant (m) (D102N) GzmH was cloned into pET-28a with an enterokinase cleavage site before N-terminal amino acids IIGG as described (L. Wang, K. Zhang, L. Wu, S. Liu, H. Zhang, Q. Zhou, L. Tong, F. Sun, and Z. Fan, manuscript in preparation). Wild-type (WT) HBx cDNA, and its mutants were cloned into the pET-28b or pcdNA-3.1 vector. The N-terminal FLAG-tagged HBx and its mutants were cloned into pSin–EF2–puro. The WT HBV 1.3 (HBV1.3WT) and the HBx-deficient HBV 1.3 (HBV1.3X-) sequences were amplified from HBV1.3 and HBV1.3X (provided by Dr. Weizsäcker, University of Freiburg, Freiburg, Germany) and cloned into pBabe–puro.

Retroviral infection and transfection

pSin–EF2–puro constructs containing WT or mutant HBx was cotransfected into 293T cells with pPAX2 and pMD2.G. Viruses were harvested at 24, 48, and 72 h posttransfection, filtered through 0.45-μm filters, and stored at −80°C. HepG2 cells were transduced at 5 PFU/cell. Similarly, pBabe–puro constructs containing HBV1.3WT or HBV1.3X- was cotransfected into 293T cells with pHIT6.0 and pCMV-VSV-G. Viruses were harvested at 24 and 48 h posttransfection. Forty-eight hours posttransduction, HepG2 cells were selected in medium containing 5 μg/ml puromycin (Sigma-Aldrich). Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Recombinant protein expression

Recombinant HBx (rHBx), GzmB, GzmM, GzmH, and mGzmH were expressed in Escherichia coli and refolded from inclusion bodies as described previously (16). GzmA and GzmK were expressed and purified as described previously (17). GST and GST-tagged p53 were expressed in a Rosetta (DE3) strain and purified with glutathione Sepharose 4B beads (Amersham Pharmacia Biotech). His-tagged p53 was purified by an Ni-NTA column.

Pulldown assay

mGzmH, HBx, or GST were covalently linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer’s instructions. Recombinant proteins were incubated with immobilized proteins at 4°C for 3 h. After five washes, the bound proteins were eluted in SDS-PAGE sample buffer and analyzed by immunoblotting.

Cytotoxicity assay

For each experiment, SLO was preactivated by 10 mM DTT at room temperature (RT) for 10 min, and its sublytic dose (40–100 ng/ml) was determined to load GzmH into target cells. PBMCs from healthy donors were purchased from the Beijing Blood Center and isolated by Ficoll-Hypaque gradient centrifugation. For generation of LAK cells, PBMCs were cultured with 1000 U/ml recombinant human IL-2 for 4 d. Cytolysis mediated by LAK cells was measured by a 6-h [51Cr]release assay. Target cells (1 × 105) were labeled with 200 μCi [51Cr] at 37°C for 1 h. After a complete wash, target cells were plated at 1 × 105 cells/well. LAK cells were added at the indicated E:T ratios. After 6 h of incubation, the supernatants were harvested, and the radioactivity was measured as previously described (18). For inhibition assay, LAK cells were pretreated with 500 nM CMA for 2 h or with 100 μM GzmH inhibitor for 1 h before incubation with target cells.

Cleavage assay

rHBx or cell lysates were incubated at 37°C for the indicated times with different concentrations of GzmH or mGzmH in 20 μl cleavage buffer (20 mM Tris-Cl [pH 7.6], 150 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2). The reactions were stopped by adding 5× SDS loading buffer. Samples were subjected to SDS-PAGE for immunoblotting (19).

Confocal microscopy

HepG2 cells were transfected with FLAG-HBX. Forty-eight hours posttransduction, cells were loaded with mGzmH plus SLO for 1 h, washed, and fixed with 4% paraformaldehyde for 30 min at RT. After being permeabilized with 0.1% Triton X-100 for 10 min, the cells were incubated with anti-FLAG mAb and anti-GzmH pAb overnight at 4°C. The treated cells were further stained with Alexa 488-conjugated donkey anti-mouse IgG or Alexa 594-conjugated donkey anti-rabbit IgG for 1 h at RT followed by staining with 5 μg/ml Hoechst 33342 for 10 min and observed using laser-scanning confocal microscopy (Olympus FV500 microscope; Olympus).

Real-time PCR

Genomic DNA was isolated by standard phenol-chloroform extraction procedure. Total RNA was isolated using TRIzol reagent and reversely transcribed with Superscript II (Invitrogen, Carlsbad, CA). Viral contents were measured in a Rotor-Gene 6000 analyzer (Corbett Research, Sydney, NSW, Australia). HBV DNA was quantified after normalizing to β-actin, whereas 18S rRNA was used as the internal control for RNA. The primers were as follows: HBV sense, 5'-CCGGTTTGGTCTAAATC-3'; HBV antisense, 5'-GGCCGGAAGTTTGTGATACG-3'; β-actin sense, 5'-CTTAGTTGGCTACCCCTTC-3'; and β-actin antisense, 5'-ACCTTCACCGTGTCAGTTTTT-3'; rRNA sense, 5'-ACCGCAGCTAGGAATGAGG-3'; and rRNA antisense, 5'-GGCTCGATGTCGAAATAACCA-3'.

Southern blot

For the purification of cytoplasmic HBV DNA, cells were washed twice with ice-cold PBS and lysed in a lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, and 1% Nonidet P-40) for 15 min at 4°C. After centrifugation for 5 min at 12,000 rpm, supernatants were added to EDTA (25 mM). Proteins were digested with 0.5 mg/ml proteinase K and 1% SDS for 2 h at 37°C. Nucleic acids were isolated by standard phenol-chloroform extraction procedure; HBV DNA was separated on a 1% agarose gel, blotted onto a nylon membrane (Hybond N+), and hybridized with a 32P-labeled full-length HBV fragment.

Adoptive transfer of NK cells to HBV carrier mice

cDNA of GzmH or mGzmH with the signal peptide and prodipeptide sequences were cloned into the pShuttle-CMV vector under the control of the CMV promoter. Recombinant adenovirus vectors (Ad-GzmH and Ad-mGzmH) expressing GzmH were purified and titered in 293FT cells, and the viral contents were transduced with Superscript II (Invitrogen, Carlsbad, CA). Viral contents were measured in a Rotor-Gene 6000 analyzer (Corbett Research, Sydney, NSW, Australia). HBV DNA was quantified after normalizing to β-actin, whereas 18S rRNA was used as the internal control for RNA. The primers were as follows: HBV sense, 5'-CCGGTTTGGTCTAAATC-3'; HBV antisense, 5'-GGCCGGAAGTTTGTGATACG-3'; β-actin sense, 5'-CTTAGTTGGCTACCCCTTC-3'; and β-actin antisense, 5'-ACCTTCACCGTGTCAGTTTTT-3'; rRNA sense, 5'-ACCGCAGCTAGGAATGAGG-3'; and rRNA antisense, 5'-GGCTCGATGTCGAAATAACCA-3'.

FIGURE 1. LAK cells repress HBV replication via the granule-mediated pathway. A, HepG2.2.15 is resistant to LAK cell-mediated apoptosis. HepG2.2.15 or K562 cells incubated with [3H]thymidine and coincubated with LAK cells pretreated with or without CMA at the E:T ratio of 30. Data are the means ± SD of three separate experiments. B, LAK cells suppress HBV replication that can be blocked by CMA. HepG2.2.15 cells were incubated with [3H]thymidine and coincubated with LAK cells pretreated with or without CMA at the E:T ratio of 30. Data are the means ± SD of three separate experiments. C, virus infectivity is confirmed by real-time PCR. Total genomic DNAs were isolated by phenol-chloroform extraction. Viral copy numbers were normalized to that of β-actin in the same sample. Virus titer of the 0 h sample without CMA treatment was set as 1. Data shown are representative of at least three separate experiments as means ± SD. The Student t test was used for statistical analysis. **p < 0.01. RC, relaxed circular; SS, single-stranded viral DNAs.
mGzmH) were prepared as described previously (21). Mouse NK cells were isolated from splenocytes through a positive NK isolation kit (Miltenyi Biotec) and infected with Ad-GzmH or Ad-mGzmH at 300 PFU/cell for 6 h. PAAV-HBV 1.2 plasmid was a kind gift from Dr. Ding-Shinn Chen (National Taiwan University and Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan). C57BL/6 mice (male, 6–8 wk old) were purchased from Vitalriver (Beijing, China). HBV carrier mice were established as described (22). GzmH- or mGzmH-overexpressed NK cells were transferred to HBV carrier mice. All animals were housed in the institute’s P2 mouse facility. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of China. The protocol was approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences (permit number SYXK2009100).

ELISA and immunohistochemistry assay

Mouse sera were collected at the indicated time points and detected for hepatitis B surface Ag (HBsAg) by an HBsAg ELISA kit (Kehua, Shanghai, China) according to the manufacturer’s instructions. The livers were fixed with 4% paraformaldehyde, embedded, sliced, and stained with rabbit anti-HBc Ab (Maxim, Fuzhou, China) followed by hematoxylin staining. Normal and HCC human liver sections were purchased from Alenabio (Xi’an, China) and stained with anti-CD56 and anti-GzmH Abs.

NK cell and GzmH analysis in peripheral blood samples

PBMCs were isolated from healthy donors, chronic HBV carriers, and HCC patients. Serum HBsAg level was determined by ELISA, and alanine transaminase level was measured by using an alanine transaminase kit (Biosino, Beijing, China). PBMCs were lysed by adding 5 × SDS loading buffer and analyzed for GzmH expression. PBMCs were fixed and permeabilized with a Fix & Perm kit (Sizhengbai, Beijing, China), stained for GzmH and CD56, followed by flow cytometry using an FACSCalibur (BD Biosciences). Peripheral blood samples of chronic HBV carriers and HCC patients were from the Third Hospital of Peking University (Beijing, China). All patients gave written informed consent, and the study was approved by the Ethical Review Board of the Third Hospital of Peking University.

Results

HBV clearance requires cytotoxic granule-mediated pathway

Two reports showed that GzmH eradicates intracellular viruses independently of induction of cytolysis (3, 11). We obtained a similar result as shown in Fig. 1A. We observed that LAK cells inhibited HBV replication without apparent cytolysis of target host HepG2.2.15 cells. In contrast, LAK cells could cause cytolytic to tumor cells (Fig. 1A). To determine whether granule exocytosis is involved in the clearance of HBV, we incubated HepG2.2.15 cells with LAK cells at the E:T ratio of 30. HepG2.2.15 cell line is a human hepatoblastoma cell line that contains an integrated HBV genome and can produce infectious HBV particles (23). Total genomic DNAs were isolated, and viral titers were measured by Southern blotting and real-time PCR at 0, 3, and 6 h after incubation. We found that HBV replication was significantly inhibited by LAK cells (Fig. 1A, 1C). CMA, an inhibitor of cytotoxic exocytosis, could remarkably block the LAK cell-mediated viral clearance. These results indicate that granule pathway participates in the clearance of HBV, and intracellular HBV clearance is not due to cytolysis of the infected cells.
**GzmH inhibits HBV replication**

To further explore which component is responsible for the viral inhibition, all five human cytotoxic Gzms (GzmA, GzmB, GzmK, GzmM, and GzmH) were loaded into HepG2.2.15 cells in the presence of a sublytic concentration of SLO (40–100 ng/ml). Our laboratory compared several delivery agents to load granzymes (Gzms) into target cells, including perforin, SLO, and a cationic lipid protein transfection agent, Pro-Ject (7, 21). We found SLO was efficiently delivered Gzms into target cells to induce cytolysis with similar kinetics of perforin delivery, which is in agreement with a previous report (6). Among all five Gzms examined, GzmH inhibited HBV replication in the most efficient way (Fig. 2A). All of the Gzms were used in the concentrations of 0.25 and 0.5 μM, and they were all enzymatic active, as detected by their synthetic substrates (Supplemental Fig. 1). We further found that GzmH disrupted replication of HBV DNA, but not RNA (Fig. 2B, 2C). The enzymatically inactive mGzmH (D102NGzmH) plus SLO did not repress viral replication (Fig. 2B). Thus, the viral clearance needs its enzymatic activity. GzmH loading into HepG2.2.15 cells did not cause apparent cytolysis (Fig. 2D), which is reminiscent of attack by LAK cells. By contrast, GzmH induced ∼50% death in K562 cells. GzmH and SLO alone or mGzmH plus SLO caused comparable death. Based on our structural analysis and peptide screening, we designed a specific inhibitor, Ac-PTSY-CMK, that only efficiently blocks the enzymatic activity of GzmH, not other Gzms or relevant enzymes (L. Wang et al., manuscript in preparation). This Ac-PTSY-CMK inhibitor was synthesized with acetylated-Pro at the P4 position and chloromethylketone (CMK)-linked Tyr at the P1 site, an irreversible inhibitor that blocks GzmH through snugly docking into the pocket of the GzmH. LAK cells were pretreated with the GzmH inhibitor before coinoculation with HepG2.2.15 cells. The GzmH inhibitor was able to block LAK cell-mediated viral clearance (Fig. 2E). These results indicate that GzmH participates in HBV clearance.

**GzmH associates with the HBx protein**

The HBx protein is a multifunctional regulator that is essential for HBV replication both in vitro and in vivo (13, 14). Because HBx is essential for HBV replication, we wanted to detect whether GzmH can proteolyze native HBx. To further verify that HBx degradation by GzmH is physiologically relevant, HBx-transduced HepG2 cells were loaded with GzmH plus SLO. GzmH was able to degrade native HBx of intact cells (Fig. 4C). GzmH and SLO alone or mGzmH plus SLO did not cause HBx degradation. NM23H1 was unchanged as a good loading control.

**GzmH cleaves HBx at Met79**

GzmH has a preference for Phe, Tyr, and Met at the P1 site (10). Based on the cleavage specificity and product sizes, potential cleavage sites were predicted. Using site-directed mutagenesis, the candidate P1 sites were mutated; that is, Phe13 to Gly (F73G-HBx), Tyr111 to Val (Y111V-HBx), Met79 to Val (M79V-HBx), Leu71 to Gly (L71G-HBx), and Leu93 to Gly (L93G-HBx), respectively. Met79 mutated to Val (M79V-HBx) completely abolished cleavage by GzmH (Fig. 4D). In contrast, other mutants did not affect the GzmH-mediated cleavage (Fig. 4D and data not shown). A variety of HBV subtypes have been identified (26). Interestingly, the Met79 residue in HBx is highly conserved among all these subtypes. WT or M79V-HBx was transfected into HepG2 cells and loaded GzmH with SLO. M79V-HBx was not proteolyzed by GzmH (Fig. 4E), whereas the WT HBx was still degraded. HBx degradation was completely blocked by the GzmH.
inhibitor at an inhibitor/effector ratio of 4:1 (Fig. 4F). Moreover, the GzmH inhibitor could significantly inhibit native HBx proteolysis mediated by LAK cells at the E:T ratio of 5 (Fig. 4G). The partial inhibition of HBx cleavage by the GzmH inhibitor may be caused by its inefficient entry or degraded by other enzymes activated by LAK cells.

**HBx-deficient HBV is resistant to clearance by GzmH or LAK cells**

We established a stable HepG2 cell line transduced with the WT HBV 1.3 (HBV1.3WT) or the HBx-deficient HBV 1.3 (HBV1.3X-). The sequence of HBx-deficient HBV 1.3 was identical to WT HBV 1.3, except for one point mutation introduced within the HBx coding region, which prevents the synthesis of HBx protein (27). The replication rate of the HBV1.3WT was 7-fold higher than that of the HBx-deficient HBV1.3X- (Fig. 5A), which is consistent with a previous report (28). HepG2-HBV1.3WT or HepG2-HBV1.3X- cells were treated with GzmH plus SLO, and the viral titers were measured. Fold decrease was calculated as the titer before GzmH loading divided by that after GzmH loading. Forty-eight hours posttransduction, HepG2 cells were co-incubated with or without LAK cells at the E:T ratio of 5. LAK cells were removed, and HepG2 cells were extracted for immunoblotting with anti-HBx Ab. NM23-H1 was used as a loading control. For the inhibition of GzmH, LAK cells were pretreated with GzmH inhibitor for 1 h prior to coincubation.

Adoptive transfer of GzmH-overexpressed NK cells facilitates HBV clearance in mice

We established an HBV-infected mouse model by hydrodynamic injection of pAAV-HBV 1.2 plasmids as described (22). The serum HBsAg levels became steady 2 to 3 wk after injection and lasted for >2 mo. To verify whether GzmH is able to facilitate HBV clearance in vivo, we isolated NK cells from the spleen of a healthy C57BL/6 mouse and transduced with Ad-GzmH or Ad-mGzmH. GzmH was expressed and its active form was processed in mouse NK cells (Fig. 6B). GzmH- or mGzmH-overexpressed

**FIGURE 4.** GzmH cleaves HBx after Met79. A, GzmH cleaves rHBx in a dose- and time-dependent manner. rHBx was incubated with different concentrations of GzmH for 120 min or with 100 nM GzmH for the indicated times. The reactions were stopped by adding 5× SDS loading buffer and visualized by Coomassie staining. B, Native HBx is degraded by GzmH. HepG2 cells transduced with HBx were lysed and treated with GzmH or mGzmH as in A and followed by immunoblotting for HBx. NM23-H1 served as a loading control. C, GzmH hydrolyzes HBx in intact cells. HepG2 cells transduced with HBx were treated with GzmH or mGzmH plus SLO at 37°C for 6 h. D, HBx is cleaved after Met79. Potential cleavage sites were mutated by site-directed mutagenesis. WT, L71G, or M79V HBx were treated with 100 nM GzmH at 37°C for 30 min and visualized by SDS-PAGE followed by Coomassie staining. E, M79V-HBx mutant is resistant to GzmH hydrolysis. HepG2 cells transduced with WT or M79V-HBx were treated with 100 nM GzmH plus SLO at 37°C for 6 h. F and G, GzmH inhibitor Ac-PTSY-CMK can block GzmH- or LAK cell-mediated HBx degradation. GzmH was pretreated with the indicated ratios of inhibitor for 30 min and then incubated with rHBx followed by Coomassie staining (F). HepG2 cells were transduced with HBx. Forty-eight hours posttransduction, HepG2 cells were co-incubated with or without LAK cells at the E:T ratio of 5. LAK cells were removed, and HepG2 cells were extracted for immunoblotting with anti-HBx Ab. NM23-H1 was used as a loading control. For the inhibition of GzmH, LAK cells were pretreated with GzmH inhibitor for 1 h prior to coincubation.

**FIGURE 5.** HBx-deficient HBV is resistant to clearance by GzmH and LAK cells. A, HBx is essential for HBV replication. HepG2 cells stably transduced with WT HBV 1.3 (HBV1.3WT) or the HBx-deficient HBV 1.3 (HBV1.3X-) were established. Viral titers were measured as above. Viral copy numbers were normalized to that of β-actin in the same sample. Viral titer of HepG2-HBV1.3-X- was set as 1. B, HBx-deficient cells (HBV1.3X-) resist clearance by GzmH. HBV1.3WT and HBV1.3X- cells were treated with GzmH plus SLO, and the viral titers were measured. Fold decrease was calculated as the titer before loading divided by that after loading. C, HBx-deficient cells (HBV1.3X-) are resistant to clearance by LAK cells. HBV1.3WT and HBV1.3X- cells were coincubated with LAK cells at the E:T ratio of 30 at 37°C for 6 h. Fold decrease was measured as in B. The Student t test was used for statistical analysis. ***p < 0.001.
NK cells were transferred to HBV carrier mice, as shown in Fig. 6A. GzmH-overexpressed NK cells facilitated clearance of HBV more effectively than that of mGzmH-overexpressed NK cells (Fig. 6C). Serum HBsAg levels were undetectable in some GzmH-overexpressed mice after 8 d transfer. HBeAg+ hepatocytes were very rare in livers of GzmH-overexpressed mice (Fig. 6D, left panel). However, more HBeAg+ hepatocytes appeared in livers of mGzmH-overexpressed mice. The average percentages of HBeAg+ hepatocytes were quantified (Fig. 6D, right panel).

Low expression of GzmH is susceptible to HBV infection
To further determine the relationship of GzmH expression and HBV infection, we isolated human PBMCs from healthy donors and chronic HBV carriers. GzmH expression was extremely low or undetectable in chronic HBV carriers (Fig. 7A). By contrast, GzmH was expressed in healthy donors. These data are representative of all the detected healthy donors (n = 22) and chronic HBV carriers (n = 17). Similar results were obtained by flow cytometry analysis (Fig. 7B). Percentages of GzmH+NK cells in chronic HBV carriers were much less than those of healthy donors (Fig. 7C). Notably, a low rate of peripheral GzmH+NK cells was found in the patients of HCC. GzmH was much less expressed in intrahepatic NK cells of HCC than those of normal livers (Fig. 7D, 7E). Taken together, low expression of GzmH predisposes individuals to HBV infection as well as HCC.

Discussion
Cytotoxic lymphocytes, including NK cells and CTLs, play important roles in viral clearance through the granule exocytosis pathway (29). GzmH is mainly expressed at a high level in NK cells (15), which suggests that GzmH may play an important role in innate immunity against pathogens or transformed tumors. We and Fellows et al. previously demonstrated that GzmH can induce apoptosis of target tumor cells using different death pathways (5, 6). Andrade et al. (3) found direct cleavage of adenosine components (DNA-binding protein and 100K) by GzmH blocked virus replication. In this study, we found that GzmH directly cleaves the viral HBx protein of HBV, leading to disruption of virus replication without cell death. Low expression of GzmH in NK cells is susceptible to HBV infection and HCC.

Because viruses are intraacellular pathogens, killing the infected host cell has been considered an ultimate pathway to eradication of the infection. The accepted dogma is that complete clearance of intraacellular viruses depends on the destruction of infected cells by NK/CTL cell-mediated cytotoxicity (30). However, two reports demonstrated that GzmH exerts antiviral activity independently of induction of cell death (3, 11). In this study, we found that eradication of HBV by LAK cells or GzmH is not dependent on induction of cytolysis either. By contrast, LAK cells or GzmH can induce cell death of target tumor cells (Figs. 1C, 2D). These suggest that eradication of different pathogens by LAK cells or GzmH might use distinct granule exocytosis pathways. Interestingly, two recent reports showed that GzmA and GzmK can induce production of proinflammatory cytokines to eliminate pathogens without induction of cytolysis (31, 32).

Chronic infection of HBV is mainly established by vertical transmission. Impaired HBV-specific immune responses cannot efficiently eliminate the infected hepatocytes (33). However, immunocompetent adults typically result in acute hepatitis, and the virus is rapidly cleared without long-term consequences (12, 34). So it is believed that both innate and adaptive immune responses are involved in the control of HBV in humans (35). In this study, we demonstrated that low expression of GzmH is susceptible to HBV infection. GzmH participates in the inhibition of viral replication. Adoptive transfer of NK cells with overexpression of human GzmH accelerates viral eradication in HBV carrier mice. Our data indicate that the granule exocytosis pathway plays a critical role in clearance of HBV. A recent clinical report showed that NK cells had faster kinetics than HBV-specific T cells in very early HBV infections (36). This observation and our data imply that innate effector NK cells play an essential role in the elimination of HBV infection. However, this notion was not achieved by NK cell- and perforin-deficient mouse strains with an HBV infection model (20). This may be because the mouse model cannot mimic the real HBV infection in humans.

Because HBV cannot infect mice, no ideal mouse models have been established to mimic genuine HBV infection in humans. GzmH is predominantly expressed in human NK cells regardless of the activation status (15). It suggests that GzmH may exert critical roles in innate immunity induced by NK cells. In this study, we found that GzmH, not other Gzms, takes part in inhibition of HBV replication, which is in agreement with clinical evidence (36). Up to date, no murine homolog has been identified for human GzmH. Murine GzmC is presumed to be a counterpart of human GzmH.

**FIGURE 6.** Adoptive transfer of GzmH-overexpressed NK cells facilitates HBV clearance in vivo. A, Schematic representation shows the experimental design. C57BL/6 mice were hydrodynamically injected with pAAV-HBV1.2 plasmid. Four weeks postinjection, serum HBsAg levels were detected by ELISA. Mice with similar HBsAg levels were randomly divided into two groups. NK cells were isolated from healthy mouse spleens,transduced with Ad-GzmH or Ad-mGzmH, and transferred to HBV carrier mice. B, Active GzmH was overexpressed in mouse NK cells. Mouse NK or HepG2 cells were transduced with Ad-GzmH, and the expression of GzmH was detected by immunoblotting. Human NK cells were used as a positive control. C and D, Adoptive transfer of GzmH-overexpressed NK cells accelerates HBV clearance. Serum HBsAg levels were analyzed at the indicated time points after transfer. For each mouse, the HBsAg level before transfer was set as 1. n = 10. C, The livers were fixed and stained for hepatitis B core Ag (HBeAg) on day 8 after transfer. Representative images from three mice in each group were shown. The average number of HBeAg+ hepatocytes was quantified for three animals for each group. Original magnification ×200. *p < 0.05, **p < 0.0001.
(37). However, HBx was not cleaved by GzmC (Supplemental Fig. 2). This suggests that the mechanisms against HBV in humans are different from HBV mouse models.

Among the proteins encoded by HBV, HBx is essential for viral replication both in vivo and in vitro (13, 14). The HBx protein is highly conserved in mammalian hepadnaviruses. HBx is a multifunctional regulator that modulates a broad spectrum of activities through direct or indirect association with host factors (38). HBx stimulates HBV replication by promoting calcium signaling (39). Whether HBx can enhance viral RNA replication is still controversial. In our study, we found that GzmH can only inhibit the replication of HBV DNA, but not replication of viral RNA (Fig. 2B, 2C). Interestingly, GzmH cleavage site Met79 is well conserved among all HBV subtypes that have been identified. Moreover, clinical studies showed that HBx harbors hot spots of mutations (40). However, Met79 was not included in any of these hot spots. High conservation implies that Met79 is critical for HBx function. Cleavage at the preserved residue by GzmH might prevent HBV from escaping through HBx mutations. Therefore, our findings demonstrate that GzmH is essential for HBV clearance, and GzmH may serve as a potential parameter for diagnosis of HBV infection and HCC.

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Disclosures
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References


