BASIC AND TRANSLATIONAL—LIVER

Blocking the Natural Killer Cell Inhibitory Receptor NKG2A Increases Activity of Human Natural Killer Cells and Clears Hepatitis B Virus Infection in Mice

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BACKGROUND & AIMS: We studied the functions of natural killer (NK) cells and the role of the NK cell inhibitory receptor (NKG2A) during hepatitis B virus (HBV) infection in patients and mice. **METHODS:** We analyzed levels of NKG2A on peripheral blood NK cells from 42 patients with active chronic hepatitis B (CHB), 31 patients with inactive CHB, and 35 healthy volunteers (controls). Five patients with CHB treated with antiviral therapy were also included to evaluate changes in NK cells after HBV titers decreased. We examined the effects of blocking antibodies against NKG2A or its ligand Qa-1 (equivalent to HLA-E in humans) in immunocompetent mice that express HBV from a plasmid and are positive for serum hepatitis B surface antigen (a mouse model of HBV infection). **RESULTS:** A higher percentage of NK cells from patients with active CHB were positive for NKG2A (38.47%) than from patients with inactive CHB (19.33%; $P < .01$) or controls (27.96%; $P < .05$). The percentage of NKG2A$^+$ cells correlated with serum viral load ($r = 0.5457$; $P < .001$). The percentage of NKG2A$^+$ cells decreased along with HBV load in patients that received antiviral therapy ($P < .05$). Blocking NKG2A interaction with HLA-E in peripheral NK cells from patients with active CHB increased their cytotoxicity in vitro. NK cells of HBV carrier mice also had higher percentages of NK cells that expressed NKG2A compared with control mice; NKG2A was likely to be up-regulated by production of interleukin-10 by hepatic regulatory CD4$^+$CD25$^+$ T cells. Blocking Qa-1 in these mice promoted viral clearance in an NK cell–dependent manner. **CONCLUSIONS: Infection with HBV increases levels of the inhibitory receptor NKG2A on NK cells in mice and humans, and reduces their ability to clear HBV. Reagents designed to block the interaction between NKG2A and HLA-E might be developed to treat CHB infection.**

Keywords: Liver Disease; Hydrodynamic Injection; IL-10; Cirrhosis.

Hepatitis B virus (HBV) infection is a serious public health problem, as it is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[1] HBV-specific CD8$^+$ T cells play an important role in controlling viral replication, and impaired CD8$^+$ T cell activity contributes to the establishment of chronic HBV infection.[2] However, few studies have focused on exploring the role of innate immune cells in chronic hepatitis B (CHB) patients, such as the liver-resident natural killer (NK) cells.

NK cells are the predominant lymphocyte subpopulation in the liver. Despite the controversy regarding NK cell frequency in CHB,[3–7] NK cells have been reported to contribute to liver inflammation in CHB patients during liver inflammatory flares.[8] Additionally, a significant decrease in NK cell activation was observed during the peak of HBV viremia as compared with the resolution phase of acute infection.[9] These data suggest that NK cell activity in CHB patients can be affected by viral load.

CHB patients can be classified according to their serologic status as immune tolerant, immune active (chronic hepatitis), or immune inactive.[10] Functionally, NK cells are controlled by the balance of activating and inhibitory NK cell receptor (NKR) signals,[11] including the activating receptor NKG2D or the inhibitory receptor NKG2A. Recently, we reported that reduced expression of the activating receptor NKG2D impaired NK cell function in immune-tolerant patients[12]; however, patients in the immune-tolerant phase are often perinatal and have normal alanine aminotransferase levels, indicating no liver injury. Additionally, NKG2D is not an ideal target for clinical blockade because it is extensively expressed on a wide variety of immune cells. We observed that the inhibitory receptor NKG2A also plays an important role in down-regulating NK cell function in immune-active patients, although its expression and mechanism are not clearly described in CHB patients of different disease stages.[4,7,13] Recently, anti-NKG2A treatment was reported to enhance NK cell ability to eliminate pathogenic T cells and to arrest autoimmune disease progression[14,15]; this suggests

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Abbreviations used in this paper: BrdU, 5-bromo-2′-deoxyuridine; CHB, chronic hepatitis B; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IL, interleukin; mAb, monoclonal antibody; NK, natural killer; NKR, natural killer cell receptor; PBMC, peripheral blood mononuclear cell.

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Table 1. Characteristics of CHB Patients and Healthy Controls

	Healthy controls	All HBV-infected patients	Inactive CHB patients	Active CHB patients
Total N	35	73	31	42
Sex (male/female), n	20/15	51/22	21/10	30/12
Median age, *y*	29	34	37	33
Median ALT (*U/L*)	NA	81	32	148
HBsAg (+/−)	0/35	73/0	31/0	42/0
HBeAg (+/−)	0/30	32/41	0/31	32/10
HBV DNA copies/mL, median (range)	Negative	1.87×10^5 (BLQ−3×10^8)	1.06×10^3 (BLQ−2×10^4)	1.90×10^7 (1.83×10^6−3×10^8)

ALT, alanine aminotransferase; BLQ, below limit of quantification; NA, not applicable.

that it might also be possible to use NKR-targeted immunotherapy to treat HBV infection.

In this study, we examined surface NKR expression in large study populations of healthy donors as well as active or inactive CHB patients. NK cells from active CHB patients expressed high levels of the inhibitory receptor NKG2A and normal levels of activating receptors as compared with healthy controls and inactive patients. In an HBV-carrier mouse model, NKG2A up-regulation on NK cells was also observed, which was induced by HBV-induced regulatory $CD4^+CD25^+$T cell−derived interleukin (IL)-10. NKG2A blockade by antibodies enhanced cytotoxicity from NK cells isolated from active CHB patients. In addition, blocking either NKG2A or its ligand Qa-1 by monoclonal antibody (mAb) treatment in vivo markedly promoted HBV clearance in HBV-carrier mice. Together, these findings suggest that NKG2A in HBV-infected patients can lead to NK cell hypoactivity, and that NKG2A blockade can enhance NK cell function as an alternative approach to promote HBV clearance in active CHB patients.

Methods

Study Population

Data reported in this study were obtained from 42 active CHB patients, 31 inactive CHB patients, and 35 healthy age- and sex-matched volunteers as described in Table 1. Active CHB patients were characterized by elevated alanine aminotransferase levels and HBV DNA levels ≥2000 IU/mL. Inactive patients were characterized by the absence of HBeAg and the presence of anti-HBe antibodies, normal alanine aminotransferase levels, and HBV DNA <2000 IU/mL.[10] Eight healthy donors were used to collect fresh peripheral blood mononuclear cells (PBMCs) that were stimulated by hIL-10 or patient serum samples. Five CHB patients given an antiviral combination treatment of recombinant interferon alfa-1b and adefovir dipivoxil were also enrolled. Informed consent was obtained from each patient, and the study was approved by the ethical committee of the University of Science and Technology of China for the protection of human subjects. All patients were negative for antibodies against hepatitis C, hepatitis D, and human immunodeficiency viruses, as well as for schistosoma.

Mice

C57BL/6 mice and BALB/c mice were purchased from the Shanghai Experimental Center, Chinese Science Academy (Shanghai, China). IL-$10^{-/-}$ and $Rag1^{-/-}$ mice (initially purchased from Jackson Laboratories, Bar Harbor, Maine) were bred in our animal center. All mice were housed in a specific pathogen-free facility and were used according to the animal care regulations of the University of Science and Technology of China.

Flow Cytometry Analysis

Blood samples were incubated with the indicated fluorescently labeled antibodies, and red blood cells were lysed with RBC lysis buffer (Biolegend, San Diego, CA). Mouse liver mononuclear cells were separated,[16] blocked, and incubated with the indicated fluorescent mAbs. HLA-E expression on K562 cells was detected by indirect fluorescence-activated cell sorting analysis, as follows. K562 cells were washed, blocked, and incubated with primary anti–HLA-E antibody (MEM-E/07; Abcam, Cambridge, MA) for 30 minutes at 4°C. Cells were then washed twice and incubated with Alexa Fluor 647−labeled secondary antibody. IL-10 expression was evaluated using an intracellular staining set (eBioscience, San Diego, CA). Freshly isolated and IL-10–stimulated PBMCs were incubated with K562 cells (effector to target ratio = 10:1) for 4 hours in the presence of an anti-CD107a antibody (BD Pharmingen, San Jose, CA) to assess NK cell degranulation.[17] All samples were analyzed by flow cytometry on a FACSCalibur cytometer using WinMDI 2.8 and FlowJo7.6.1 software.

NK Cell-Mediated Cytotoxicity Assay

PBMCs pretreated with 10 μg/mL anti-NKG2A (Z199; Beckman Coulter, Brea, CA) or mouse IgG2b (BD Pharmingen) were incubated with ^{51}Cr-labeled K562 cells at different effector to target ratios. ^{51}Cr released into supernatant was assessed and percent lysis was calculated as described previously.[12]

Animal Studies

A previously described immunocompetent mouse model was used to model chronic HBV infection in humans.[18] In brief, 4- to 6-week-old mice were hydrodynamically injected with 6 μg HBV plasmid DNA, and mice positive for serum $HBsAg^+$ were selected 2 to 4 weeks later. Either an anti-NKG2A/C/E antibody (20D5; eBioscience) or an anti–Qa-1 antibody (6A8.6F10.1A6; BD Pharmingen) was intravenously administered (100 μg) twice to block the NKG2A−Qa-1 interaction. Alternatively, anti-AsGM1 (Wako Pure Chemicals Industries, Osaka, Japan) was administered intravenously (50 μg) to deplete NK cells. Rat IgG2a or rat IgG1 isotype control antibodies were administered to control groups, and serum samples were collected and analyzed at the indicated time points.

Regulatory $CD4^+$ T cells were separated using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch

Gladbach, Germany). A total of 10^4 $CD4^+CD25^+$ T cells from HBV-carrier C57BL/6 WT or IL-$10^{-/-}$ mice were injected intravenously into 6- to 8-week-old $Rag1^{-/-}$ mice. In some recipients, 300 μg anti-mouse CD210 (IL-10R) (Biolegend, San Diego, CA) or rat IgG1, κ (Biolegend) was administered to interrupt IL-10 signaling; recipient mice were euthanized and analyzed 1 week later. For in vitro experiment, $5 \times 10^5 CD4^+CD25^+$ T, $CD4^-$ T, and $CD4^+CD25^-$ T cells from HBV-carrier mice were cocultured with 1×10^5 magnetic-activated cell sorting–separated NK cells from naïve mice in IL-15–free 1640 medium.

In Vivo 5-Bromo-2′-Deoxyuridine Incorporation

C57BL/6 mice were hydrodynamically injected with pAAV/HBV1.2 or phosphate-buffered saline control and were intraperitoneally injected with 1 mg 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St Louis, MO); all mice were treated with 0.8 mg/mL BrdU in drinking water for 2 weeks. Liver lymphocytes were separated and $BrdU^+$ cell frequency was evaluated according to the FITC BrdU Flow Kit instructions (BD Pharmingen).

Detection of HBsAg and MHBV DNA in the Serum

Serum HBsAg levels were assessed using a commercial kit to quantify HBsAg (Beijing North Institute of Biological Technology, Beijing, China). A commercial real-time polymerase chain reaction kit was used to evaluate serum HBV DNA levels (Da An Biological Science Co. Ltd., Guangzhou, China).

Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded liver tissue were stained with a rabbit polyclonal anti–hepatitis B core antigen (HBcAg) antibody (DAKO, Glostrup, Denmark) overnight at 4°C. Slides were subsequently incubated with ImmPRES anti-rabbit Ig (Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes, stained with peroxidase substrate 3,3′-diaminobenzidine chromogen (Vector Laboratories), and counterstained with hematoxylin.

Statistical Analysis

The Kruskal-Wallis test was used to evaluate the differences among more than 2 groups; these values are designated as "Pk" in the figures. The Mann-Whitney *U* test, paired *t* test, and unpaired *t* test were used to compare variables between 2 groups. A correlation analysis was performed using nonparametric correlation (Spearman). Calculations were performed using GraphPad Prism version 4.00 (GraphPad Software, Inc, San Diego, CA).

Results

Frequency of Circulating $NKG2A^+$ NK Cells Is Elevated in Active CHB Patients

To evaluate NK cell frequency in freshly isolated PBMCs from healthy controls as well as from inactive and active CHB patients (whose clinical profiles are outlined in Methods), $CD3^-CD56^+$ NK cells were analyzed by fluorescence-activated cell sorting. NK cell percentage in PBMCs was significantly elevated in inactive CHB patients (mean, 19.55%, n = 31) compared with active CHB patients (mean, 13.61%; $P = .019$, n = 42) and healthy controls (mean, 13.98%; $P = .0433$, n = 35) (Supplementary Figure 1*A, B*). The number of circulating NK cells negatively correlated with viral load among all CHB patients ($r = -0.3623$; $P = .0383$) (Supplementary Figure 1*C*), suggesting that human NK cells respond to HBV infection at lower viral titers. This lower NK cell frequency in active CHB patients could either be the cause of, or result from, higher viral titers.

Because NK cells are regulated by the balance of signals between inhibitory and activating NKRs,[19] we investigated the expression pattern of several receptors. Although a marked increase in $NKG2A^+$ NK cell frequency was observed in active CHB patients (mean, 38.47%, n = 42) compared with healthy controls (mean, 27.96%; $P < .05$, n = 35) (Figure 1*A, B*), which is consistent with a previous report,[7] it was surprisingly decreased in inactive CHB patients (mean, 19.33%; $P < .01$, n = 31) (Figure 1*A, B*). More importantly, the NKG2A mean fluorescent intensity was almost double in active CHB patients than in healthy donors (123.3 vs 76.87; $P < .01$) or in inactive CHB patients (123.3 vs 72.92; $P < .001$) (Figure 1C). Considering that the total NK cell frequency decreased (Supplementary Figure 1) and the expression of $NKG2A^+$ on NK cells increased (Figure 1*A, B*) in active CHB patients, we compared the frequency and number of $NKG2A^+$ NK cells in total PBMCs from active CHB patients with healthy donors and inactive patients; both the frequency (mean, 5.43 vs 3.41 or 3.61; $P = .0251$; $P = .016$, respectively) and total number (5.831×10^4 vs 2.55×10^4 or 3.4×10^4 cells/mL; $P = .012$, $P = .082$, respectively) of $NKG2A^+$ NK cells in PBMCs were increased (Figure 1*D, E*), and the frequency of $NKG2A^+$ NK cells positively correlated with serum viral loads ($r = 0.5457$; $P = .0004$) (Figure 1F). No difference was found in the expression of other major NK cell inhibitory (CD158a, CD158b, KIR2DL3, and KIR3DL1) or activating (NKG2D, NKG2C, NKp30, and NKp46) receptors (Supplementary Figure 2).

$NKG2A^+$ NK Cells Are Reduced in Active CHB Patients After the Decline of HBV Viral Titers After Conventional Antiviral Therapy

Because NKG2A expression on NK cells correlated with HBV titers, we evaluated NKR expression 1 week after a 2-week antiviral treatment of adefovir dipivoxil plus recombinant interferon alfa in 5 CHB patients. All patients exhibited a rapid decline in NKG2A expression during the course of treatment in both mean fluorescent intensity and frequency (Figure 2*A, B*), although the difference in NKG2A frequency was not statistically significant, likely because the NKG2A frequency in a single patient declined too rapidly (from 57.59% to 7.94%). In addition, decreased serum HBV load after antiviral treatment correlated with this decline in inhibitory NKG2A expression (Figure 2*C*), but not expression of the activating NKG2D and NKG2C receptors (Supplementary Figure 3), implying that the balance between activating and inhibitory receptors reversed in CHB patients during antiviral therapy to favor NK cell activation via loss of NKG2A-mediated inhibitory signals.

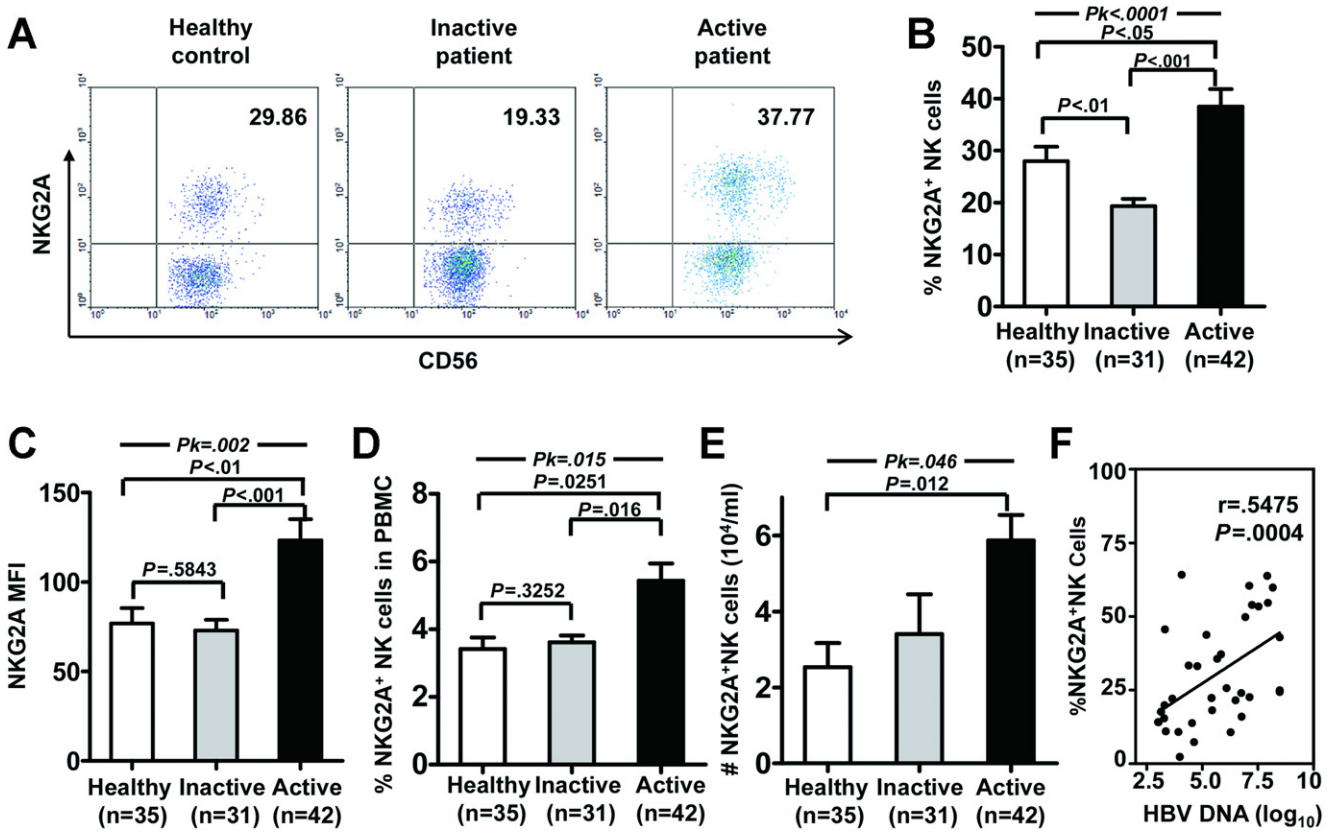

Figure 1. Active CHB patients harbor an elevated frequency of circulating NKG2A$^+$ NK cells. (*A*) Representative dot plots show NKG2A expression on peripheral NK cells in a healthy control, an inactive patient, and an active patient. The indicated numbers show the percentage from 1 representative patient. The summarized data shows the NKG2A$^+$ frequency within NK cells (*B*), NKG2A mean fluorescent intensity (*C*), NKG2A$^+$ NK cell frequency in PBMC (*D*), and total NKG2A$^+$ NK cell number (*E*) in the 3 groups. The Pk values from the Kruskal-Wallis test and *P* values from the Mann-Whitney *U* test are shown. (*F*) Correlation between NKG2A$^+$ cell frequency and viral load in all CHB patients was calculated by nonparametric correlation (Spearman).

Hepatic Regulatory CD4$^+$ T Cell-Derived IL-10 Increases NKG2A Expression on Murine NK Cells During Chronic HBV Infection

An immunocompetent mouse model for human chronic HBV that expresses HBV viral replication intermediates, transcripts, and proteins in liver tissues was used to examine HBV regulation of NKG2A expression and explore the underlying mechanism.[18] Mice hydrodynamically injected with HBV plasmid were evaluated 2 weeks later for NKG2A expression on the surface of liver-resident and PBMC NK cells. NKG2A expression on liver NK cells (mean fluorescent intensity, 13.7 vs 10.7; P = .019) and NKG2A$^+$ NK cell frequency (60% vs 53%; P = .014) were enhanced in HBV-carrier compared with blank vector-injected mice (Figure 3*A*). NKG2A expression on peripheral blood NK cells was also significantly increased in HBV-carrier mice, although there was no obvious increase in NKG2A frequency (Figure 3*B*). Interestingly, C-X-C chemokine receptor type 6 expression on hepatic but not spleen NKG2A$^+$ NK cells was higher in HBV-carrier mice than in control mice. Because C-X-C chemokine receptor type 6 helps to retain NK cells in the liver,[20] the increase in this chemokine receptor might explain the liver tropism of NKG2A$^+$ NK cells during HBV infection (Supplementary Figure 4).

As serum HBsAg in some HBV-carrier mice declined during a 7-week period to become HBsAg-negative, NKG2A expression on peripheral blood NK cells in mice concurrently decreased compared with mice that remained HBsAg-positive (mean fluorescent intensity, 13.4 vs 11; P = .02; frequency 42% vs 38%; P = .001) (Figure 3*C*) similar to the decreased NKG2A expression observed in human CHB patients treated with antiviral therapy (Figure 2). These results collectively indicated that NKG2A expression increases on NK cells in HBV-carrier mice, which is similar to what we observed in human active CHB patients (Figure 1).

Unlike C57BL/6 mice, BALB/c mice mount an effective immune response against HBV and cannot develop persistent HBV infection.[18] Interestingly, NK cells in BALB/c mice exhibit very low NKG2A expression, suggesting that they are not under NKG2A-mediated inhibition, and HBV infection cannot up-regulate NKG2A expression (Figure 3*D*). Surprisingly, expression of the activating NKG2D receptor is also lower in BALB/c than in C57BL/6 mice (Figure 3*D*). Because BALB/c are observed to clear HBV infection more effectively than B6 mice, our data argue that lower NKG2A expression on NK cells, but not of NKG2D, might at least partially explain this phenotype.

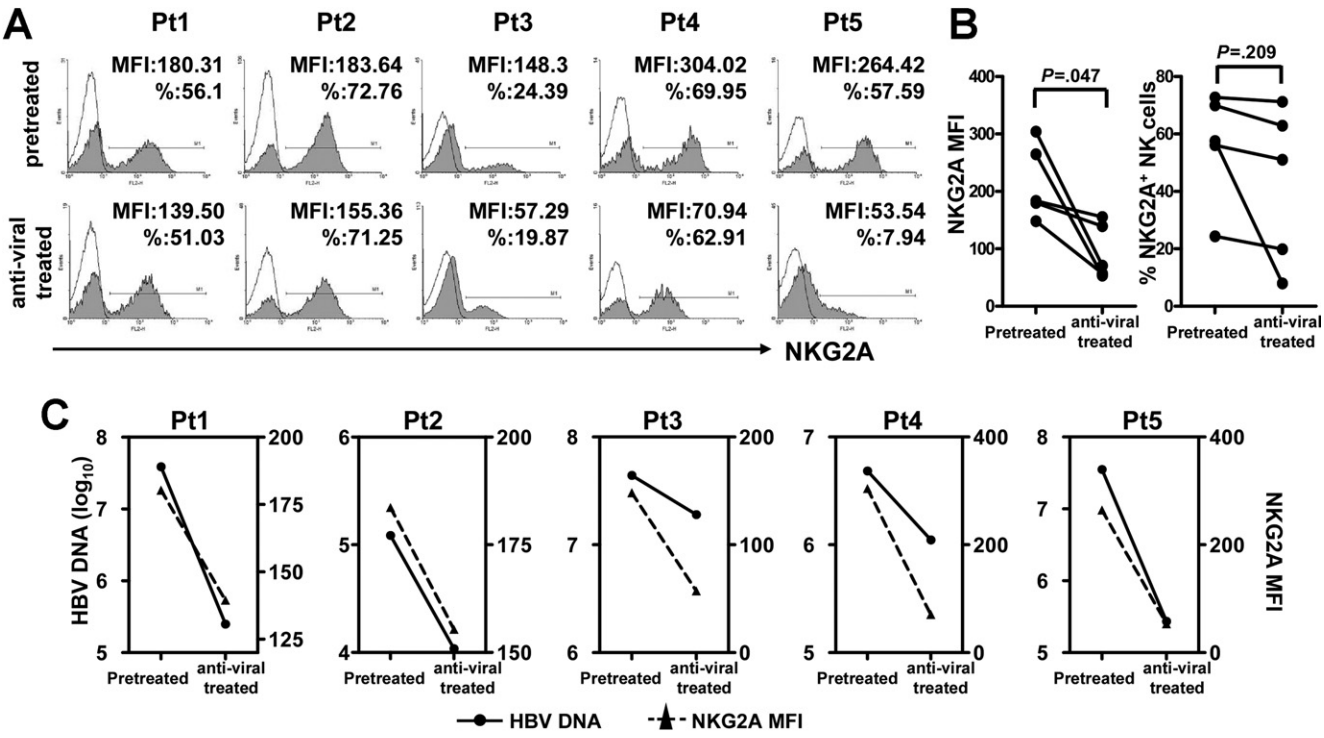

Figure 2. Antiviral treatment reduces NKG2A expression on NK cells in active CHB patients. Five active CHB patients were treated with antiviral therapy as described in the Methods. NKG2A expression on peripheral NK cells was analyzed by fluorescence-activated cell sorting. (*A*) The mean fluorescent intensity (MFI) and NKG2A$^+$ NK cell frequency from pretreated and treated CHB patients are shown. (*B*) Data from (*A*) were compiled and analyzed for significance by the paired *t* test. (*C*) Serum HBV DNA copy number was determined for each antiviral-treated patient, and its relationship to NKG2A MFI was assessed.

Cytokines can induce NKG2A expression on NK cells and cytotoxic T lymphocytes.[21, 22] We observed that serum from CHB patients did not alter NKG2A expression on NK cells harvested from peripheral blood of healthy donors, even though it could inhibit cytotoxic function (Supplementary Figure 5*A, B*). Neither cytokines nor HBV virus particles in the serum could induce NKG2A up-regulation. There are at least 2 possible explanations for not observing NKG2A up-regulation, including NKG2A$^+$ NK cells were not induced, but NKG2A$^-$ NK cells were selectively deleted and NKG2A induction might occur specifically in the liver but not in the peripheral blood. To distinguish between these possibilities, we tested the proliferative capacity of different lymphocyte subsets using BrdU incorporation assay in our mouse model. Although NKG2A$^+$ NK cells incorporated more BrdU in HBV-carrier mice than in control mice (Figure 4*A*), no significant increase was observed in NKG2A$^-$ NK, CD4$^+$ T, or CD8$^+$ T cell proliferation (Figure 4*A*), suggesting that NKG2A$^+$ NK cells selectively expanded.

We also found that liver lymphocytes from HBV-carrier, but not naïve, mice induced NKG2A expression on NK cells on transfer to Rag1$^{-/-}$ recipient mice (Figure 4*B*). To identify which liver-resident lymphocyte subset induced NKG2A up-regulation, we cocultured magnetic-activated cell-sorted CD4$^-$, CD4$^+$CD25$^-$ T, and CD4$^+$CD25$^+$ T cells from HBV-carrier or control mice with purified NK cells in vitro for 3 days; only hepatic CD4$^+$CD25$^+$ T cells from HBV-carrier mice induced NKG2A expression in vitro (Supplementary Figure 5*C*). Examining the cytokine profile of these cells, we found that intracellular IL-10 was enhanced in CD4$^+$CD25$^+$ T cells from HBV-carrier mice (Figure 4*C*) and included both Foxp3$^+$ and Foxp3$^-$ cells, the so-called regulatory CD4$^+$ T cells (data not shown). These hepatic regulatory CD4$^+$ T cells induced NKG2A upregulation in vivo, and an IL-10R blocking antibody reversed this process (Figure 4*D*), suggesting that IL-10 was required. In order to test whether IL-10 from regulatory CD4$^+$ T cells was required, and to exclude the influence of other IL-10–secreting cells, such as Kuppfer cells or DCs, we transferred regulatory CD4$^+$ T cells from HBV-infected C57BL/6 or IL-10$^{-/-}$ mice to Rag1$^{-/-}$ recipient mice. IL-10$^{-/-}$ regulatory CD4$^+$ T cells lost the ability to induce NKG2A expression on recipient NK cells (Figure 4*E*), suggesting that IL-10 from hepatic regulatory CD4$^+$ T cells derived from HBV-carrier mice induce NKG2A up-regulation on NK cells. Indeed, IL-10 was sufficient to induce NKG2A, as NKG2A expression increased and NK cell function was impaired on hIL-10 stimulation of hPBMCs from healthy donors (Supplementary Figure 5*D*). In addition, NK cells express IL-10R,[23] and increased NKG2A expression was also observed in purified NK cells cultured with hIL-10 (Supplementary Figure 5*E*). The results collectively indicate that hepatic regulatory CD4$^+$ T cell-derived IL-10 is responsible for

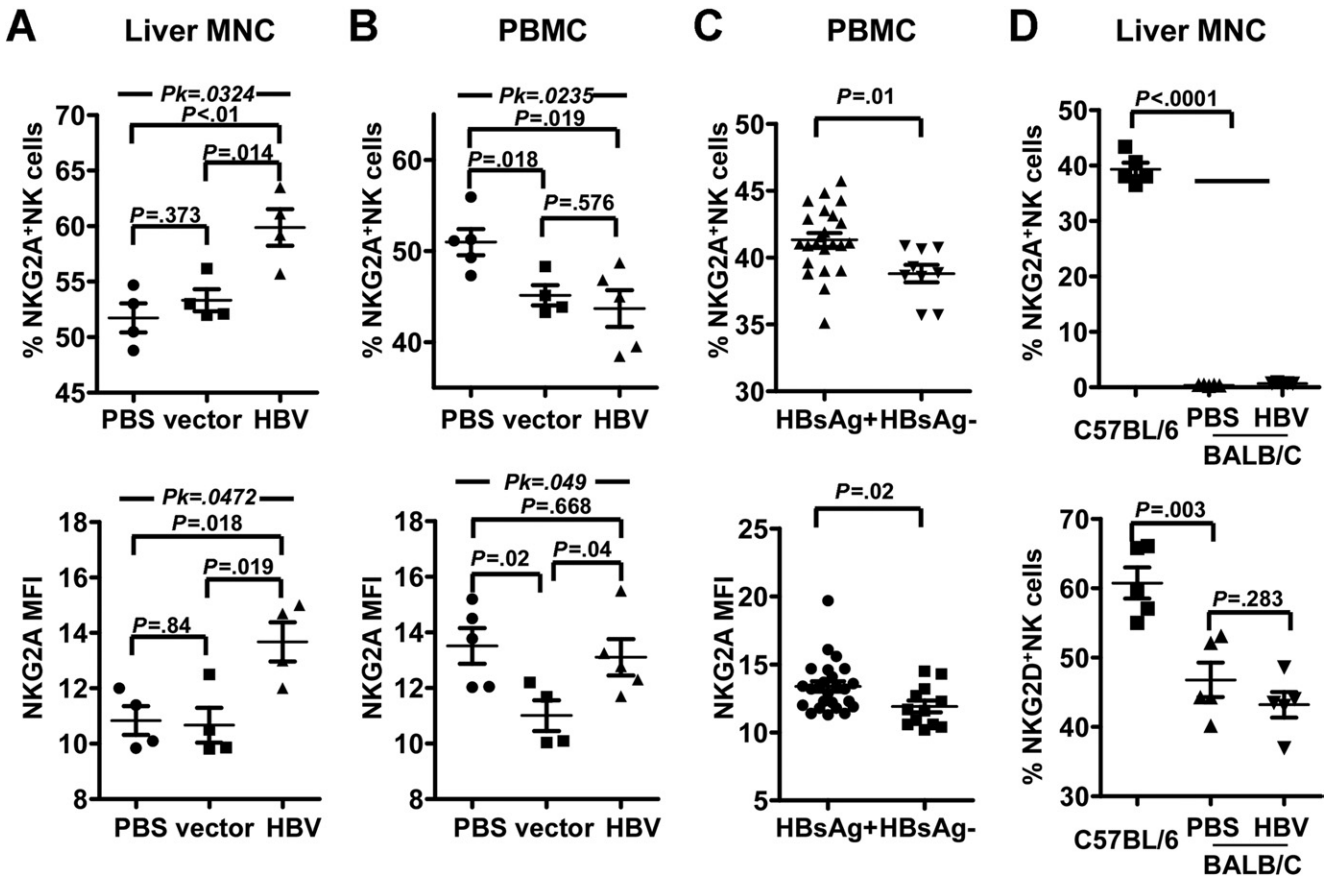

Figure 3. NKG2A expression is increased on circulating and hepatic NK cells in HBV-carrier mice. C57BL/6 mice were hydrodynamically injected with phosphate-buffered saline (PBS), pAAV/blank plasmid (vector), or pAAV/HBV1.2 plasmid (HBV). Two weeks after injection, liver mononuclear cells (MNCs) (*A*) and PBMCs (*B*) were isolated, and NKG2A expression on NK cells was analyzed by fluorescence-activated cell sorting (FACS). (*C*) Seven weeks after pAAV/HBV1.2 plasmid injection, NKG2A expression on peripheral NK cells from mice that had cleared the infection (serum HBsAg-negative) and from HBsAg-positive HBV-carrier mice were analyzed FACS. (*D*) BALB/c mice were hydrodynamically injected with PBS or pAAV/HBV1.2 plasmid (HBV). Two weeks later, NKG2A and NKG2D expression on hepatic NK cells from these BALB/c mice, as well as from normal C57BL/6 mice, were analyzed by FACS. Pk values from the Kruskal-Wallis test and *P* values from the Mann-Whitney *U* test are shown.

inducing NKG2A expression in CHB patients and HBV-carrier mice.

Blocking NKG2A Recognition In Vitro Augments NK Cell Cytotoxicity from Active CHB Patients

NKG2A inhibition is dependent on interaction with its ligand, HLA-E. Using a ^{51}Cr release assay, we cocultured PBMCs from CHB patients with HLA-E−expressing target K562 cells (Figure 5*A*) in the presence of an anti-NKG2A mAb. Our results showed that blocking NKG2A significantly enhanced K562 cell lysis by NK cells from active CHB patients, which had increased expression of NKG2A, but not by NK cells from inactive patients or healthy controls (Figure 5*B, C*). In fact, because the overall specific lysis of all 3 groups was rather small, more additional readouts such as interferon gamma of NK cells might help to further evaluate NK cells' cytotoxicity after blocking NKG2A. Because of the limitations in obtaining enough samples from human subjects, these experiments were not performed. These results demonstrate that NKG2A is critical for NK cell function during HBV infection, and blocking NKG2A can reverse HBV-mediated inhibition of NK cell function in active CHB patients.

NKG2A Blockade Reverses NK Cell Tolerance to HBV Infection in HBV-Carrier Mice

Three weeks after hydrodynamically injecting HBV plasmid, mice that remained HBsAg-positive were used to test whether anti-NKG2A mAb treatment affected NK cell tolerance during HBV infection. Serum HBsAg levels (531 vs 1298 ng/mL; $P < .001$) and viral load (9.4×10^3 vs 1.19×10^5; $P < .001$) were markedly reduced in anti-NKG2A–treated compared with control mice, respectively (Figure 6*A, B*). The effects of anti-NKG2A mAb treatment depended on the presence of NK cells, as the therapeutic effect was lost on NK cell depletion (Figure 6*A, B*). To further explore how blocking NKG2A signals can reverse NK cell tolerance, an anti−Qa-1 (the NKG2A ligand in mouse equivalent to HLA-E in humans) mAb was administered. Similarly, we observed markedly reduced HBsAg levels (344 vs 1112 ng/mL; $P < .02$) and HBV load (7.15×10^3 vs 2.37×10^5; $P < .021$) (Figure 6*C, D*) in the anti-Qa-

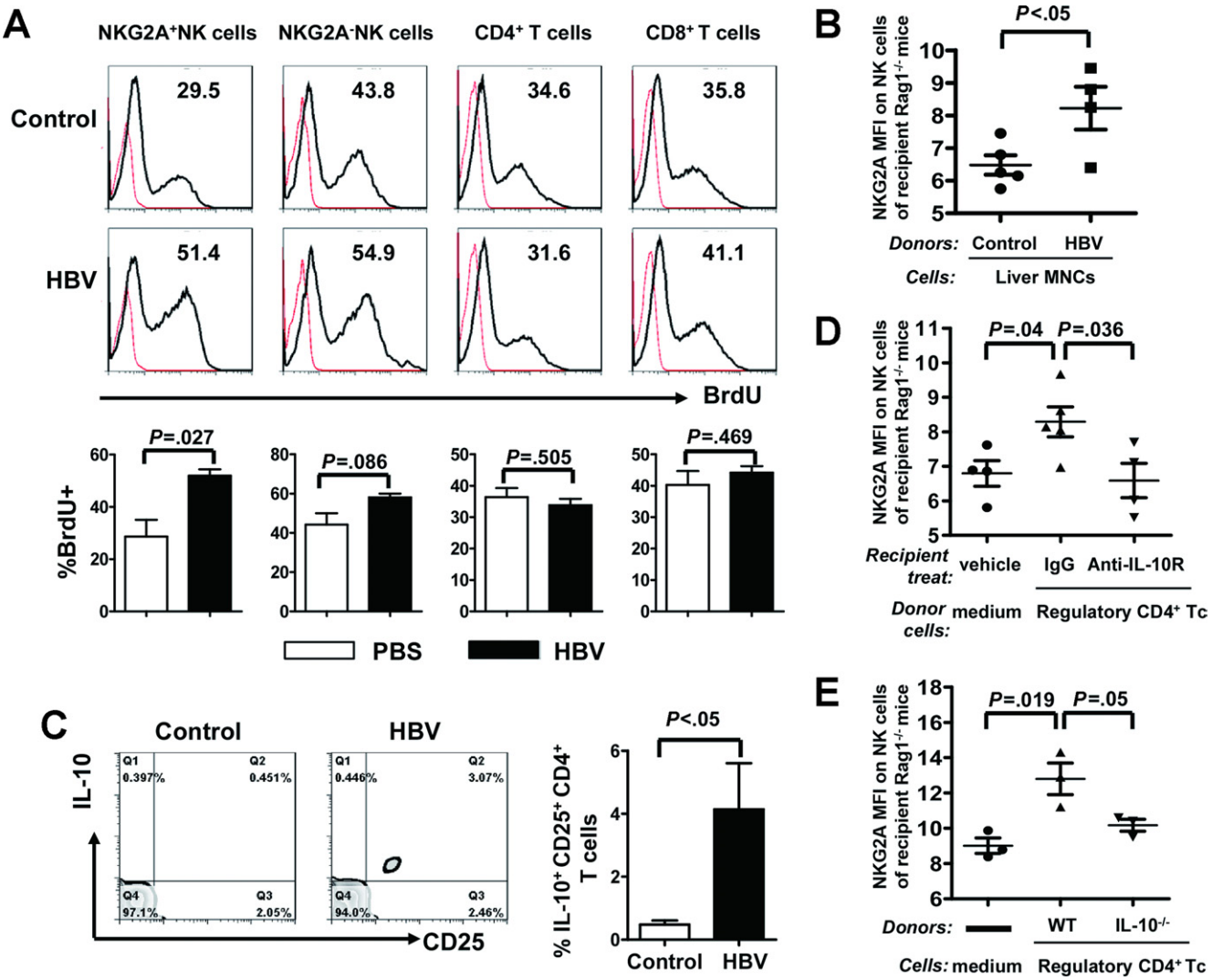

Figure 4. Hepatic regulatory CD4+ T cell-derived IL-10 induces NKG2A expression on NK cells during chronic HBV infection. (*A*) C57BL/6 mice hydrodynamically injected with PBS (Control) or pAAV/HBV1.2 (HBV) received BrdU as described in the Methods. BrdU+ cell frequency within NKG2A+ NK, NKG2A− NK, CD4+ T, and CD8+ T cells were detected by fluorescence-activated cell sorting (FACS); both the frequency and the cumulative data are shown. (*B*) Liver mononuclear cells (MNCs) from HBV-carrier or control naïve mice were transferred intravenously to Rag1−/− mice, and NKG2A expression on liver NK cells in recipient Rag1−/− mice was analyzed by FACS 1 week later. NKG2A mean fluorescent intensity is shown. (*C*) Liver MNCs from HBV-carrier or control naïve mice were stimulated with phorbol myristate acetate/ionomycin mitogens for 4 hours, and intracellular IL-10 expression was assessed by FACS. (*D*) CD4+CD25+ T cells purified from liver MNCs of HBV-carrier C57BL/6 mice were transferred intravenously into Rag1−/− mice that also received an intraperitoneal injection of anti−IL-10R or control IgG antibodies. NKG2A expression on hepatic NK cells of recipient mice was assessed by FACS 1 week later. (*E*) Liver CD4+CD25+ T cells purified from C57BL/6 and IL-10−/− mice hydrodynamically injected with pAAV/HBV1.2 were transferred to Rag1−/− mice and analyzed similar to (*D*). *P* values were calculated using an unpaired *t* test.

1−treated group compared with control mice, respectively, and the effects of anti−Qa-1 treatment also depended on the presence of NK cells (Figure 6*C, D*). HBcAg levels were also decreased in the livers of anti-Qa-1–treated HBV mice (Figure 6*E*). Because T or B cells could potentially express NKG2A, HBV-carrier Rag1−/− mice were tested to confirm that NKG2A exclusively expressed on NK cells was required for NK cell tolerance; indeed, anti-NKG2A treatment markedly reduced serum HBsAg levels in Rag1−/− mice (Figure 6*F*), indicating a direct role for NKG2A in NK cell tolerance in the context of HBV infection.

Discussion

During chronic HBV infection, the immune response against the virus induces dynamic alterations in viral load and liver inflammation. Recent evidence demonstrated that the antiviral effects of NK cells against pathogens such as herpesviruses, influenza, and ectromelia poxvirus are critical for host defense[24–26]; however, the precise role of NK cells in anti-HBV immunity remains unknown. Recently, we found that immune-tolerant patients have lower NK cell function due to down-regulation of the activating NKG2D receptor.[12] Immune-tolerant patients, who are often infected with HBV via perinatal

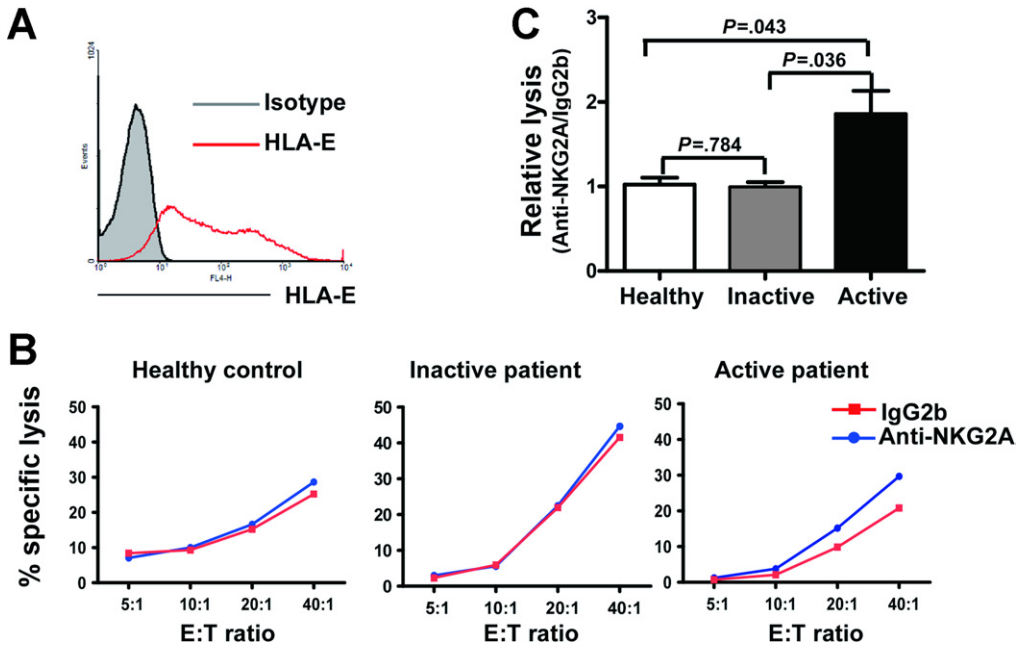

Figure 5. NKG2A blockade enhances NK cell cytotoxicity. (*A*) Fluorescence-activated cell sorting analysis of HLA-E (NKG2A ligand) expression on K562 cells. (*B*) PBMCs isolated from healthy controls, inactive patients, and active patients were analyzed for the spontaneous cytolytic activity of NK cells against target K562 cells in the presence of anti-NKG2A or control IgG2b (10 μg/mL). (*C*) The change in cytolytic activity upon NKG2A blockade (anti-NKG2A/IgG2b, effector to target = 20:1) is shown (5 samples/group). *P* values were calculated using an unpaired *t* test.

transmission, represent the early stage of NK cell tolerance without any liver injury; however, the role of NK cells needs to be understood for active CHB patients who have immune-induced liver injury due to nonresolving inflammation. In this study, we observed NK cell impairment in active CHB patients and determined that NKG2A up-regulation on NK cells by HBV-induced regulatory $CD4^+$ T cell-derived IL-10 is the underlying mechanism, which is different from the NKG2D down-regulation mechanism observed in immune-tolerant CHB patients.

Although we show in our study that the high viral replication levels in CHB patients can result from NK cell dysfunction, the mechanism for this process is currently unknown. It is possible that HBV-infected hepatocytes or other infected liver cells might play critical roles in creating a tolerogenic liver microenvironment to "re-educate" or silence NK cells under conditions of high viral replication. Other groups reported that, in a healthy state, intrahepatic IL-10 has an important role to locally maintain NKG2A expression on NK cells in the liver.[23] Some studies in human CHB patients have shown that IL-10 expression is significantly enhanced when HBV titers increase[27,28] and that IL-10 might suppress interferon gamma production by NK cells.[29] Importantly, previous studies indicate that regulatory $CD4^+CD25^+$ T cells increase in the peripheral blood and liver of CHB patients and secrete a marked amount of IL-10 that correlate to the virus load.[30–32] In the present study, our data suggest that IL-10 is a candidate cytokine to up-regulate NKG2A on NK cells in CHB patients and that hepatic regulatory $CD4^+$ T cells can produce IL-10 (Figure 4).

In active CHB patients undergoing combination antiviral therapy of recombinant interferon alfa-1b and adefovir dipivoxil, we observed that NKG2A expression on NK cells rapidly declined and was accompanied by decreased serum viral load (Figure 2), likely due to both components of the combination treatment. In addition to direct antiviral activity,[33] interferon alfa treatment alone has been reported to stimulate expression of the activating receptor NKG2D and inhibit expression of the inhibitory receptor NKG2A on NK cells in vivo within several hours of administration.[34,35] On the other hand, adefovir dipivoxil treatment alone (without interferon alfa) decreased regulatory T cells in CHB patients,[36] which were found to induce NKG2A expression in the present study. Therefore, although our experiments cannot conclusively determine whether reducing HBV viral titers was responsible for decreasing NKG2A expression in the later phase of interferon alfa therapy, our data confirmed that there was an interaction between HBV virus load and NKG2A expression (Figure 2). Our data further show that blocking NKG2A inhibition might have at least partially enhanced HBV-inhibited NK cell function in vitro (Figure 5) and reduced serum viral DNA, HBsAg, and HBcAg levels in vivo in an NK cell-dependent manner in HBV-carrier mice (Figure 6). Together these results suggest that blocking NKG2A signaling is a promising alternative approach to clear HBV in CHB patients.

NK cell–based immunotherapy is becoming an increasingly practical way to eliminate tumor cells and virus-infected cells in treated patients,[37,38] and its therapeutic efficacy correlates with NK cell frequency and function.[39] Anti-NKG2A treatment reportedly enhances NK cell ability to eliminate pathogenic T cells and to arrest autoimmune disease progression.[14,15] Treatment with interferon alfa and nucleotide analogues, the most common approach used to treat CHB, can generally inhibit HBV replication but does not fully eradicate HBV. In our study, blocking the inhibitory NKG2A receptor recovered human HBV-impaired NK cell function and also contributed to reducing serum viral DNA and HBsAg levels in HBV-carrier mice in an NK cell-dependent manner; moreover,

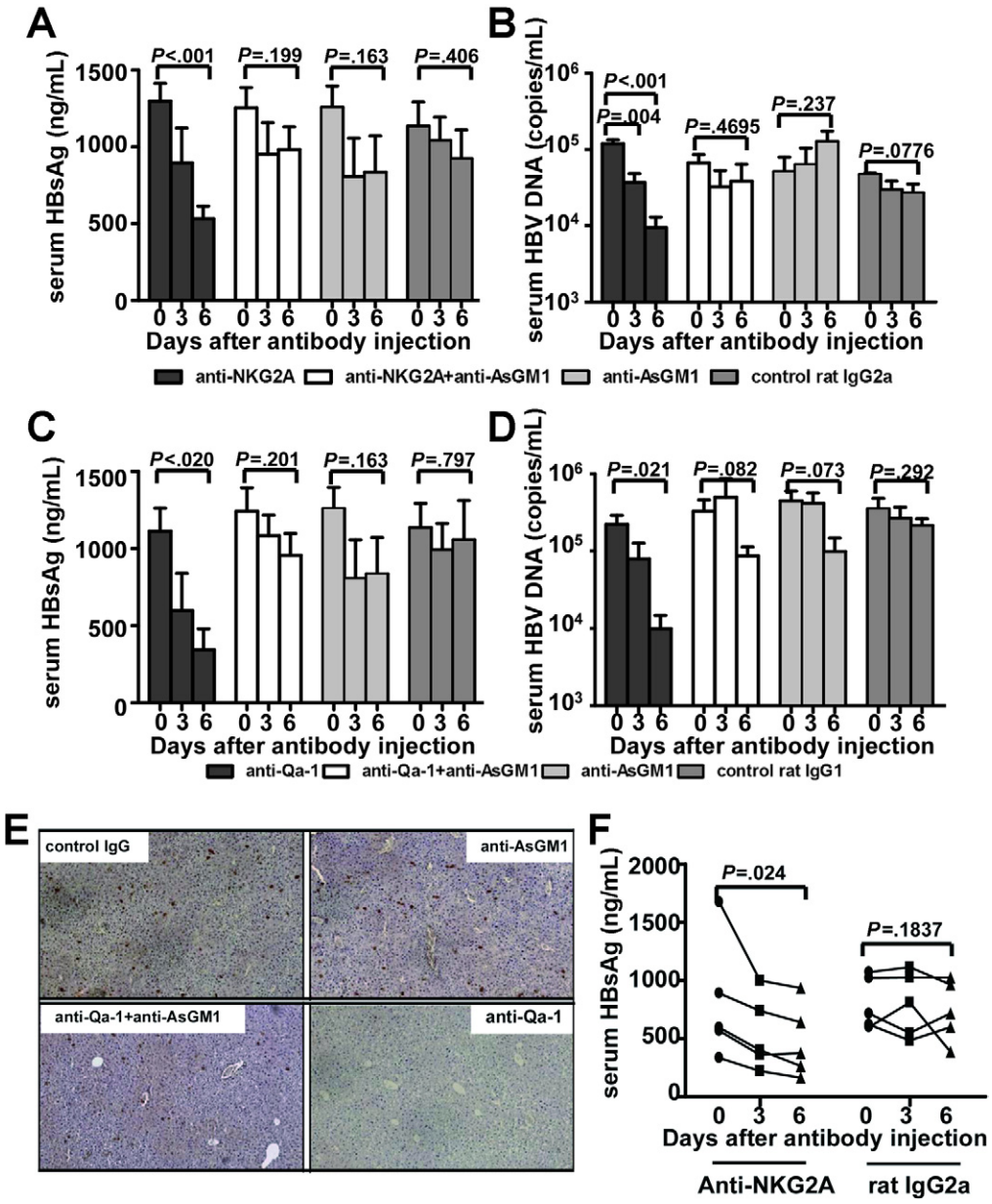

Figure 6. NKG2A blockade augments the antiviral activity of NK cells. After HBV-carrier mice were established, anti-NKG2A/C/E (20D5) or anti−Qa-1 (6A8.6F10.1A6) was intravenously injected (100 μg/mouse) twice, allowing for 3 days between injections. Control mice received rat IgG2a or IgG1. To deplete NK cells, anti-AsGM1 antibody was administered (50 μg/mouse) 1 day before anti-NKG2A or anti−Qa-1 administration. Serum samples were obtained from 8 mice/group after antibody injection. Longitudinal analysis of HBsAg levels (*A, C*) and viral loads (*B, D*) was performed on the serum of mice treated with the different antibodies. *P* values were calculated using an unpaired *t* test. (*E*) Immunohistochemical staining for HBcAg in the hepatocytes of HBV-carrier mice treated with the indicated antibodies (original magnification: 100×). (*F*) HBV-carrier Rag1$^{-/-}$ mice were established and treated with anti-NKG2A/C/E (20D5) or rat IgG2a according to the procedure described here (*A*). *P* values were calculated using a paired *t* test.

HBcAg, an indicator of active viral replication, was almost undetectable in liver tissues after blocking NKG2A. Therefore, blocking NKG2A recognition might reverse HBV-induced immune tolerance in CHB patients.

In conclusion, we initially observed that active CHB patients exhibited increased NKG2A expression on NK cells. This NKG2A up-regulation functionally inhibited NK cells, as NKG2A blockade markedly enhanced cytotoxicity in NK cells from active CHB patients. In addition, NKG2A mAb blockade in vivo markedly promoted HBV viral clearance in HBV-carrier mice, suggesting that NKG2A blockade has considerable potential to treat CHB by reversing NK cell hypoactivity. Further investigation will be required to characterize the mechanisms underlying how NKG2A expression is regulated as well as its impact on the immune pathogenesis of CHB.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.10.039.

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Conflicts of interest

The authors disclose no conflicts.

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