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Activated γδ T cells exhibit cytotoxicity and the capacity for viral clearance in patients with acute hepatitis B

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ABSTRACT

γδ T cells are a unique population of lymphocytes that have regulatory roles in patients with chronic hepatitis B (CHB); however, their role in acute hepatitis B (AHB) infection remains unclear. Phenotype and function of γδ T cells were analyzed in 29 AHB patients, 28 CHB patients, and 30 healthy controls (HCs) using immunofunctional assays. Compared with HCs and CHB patients, decreased peripheral and increased hepatic γδ T cells were found in AHB patients. Increased hepatic γδ T cells in AHB patients were attributed to elevated hepatic chemokine levels. Peripheral γδ T cells exhibited highly activated and terminally differentiated memory phenotype in AHB patients. Consistently, peripheral γδ T cells in AHB patients showed increased cytotoxic capacity and enhanced antiviral activity which was further proved in longitudinal study. Activated γδ T cells in AHB patients exhibited increased cytotoxicity and capacity for viral clearance associated with liver injury and the control of infection.

1. Introduction

Hepatitis B virus (HBV) is one of the most endemic pathogens. Approximately 250 million people worldwide suffer from its persistent infection [1]. There is considerable heterogeneity in the clinical manifestations and outcomes between acute and chronic HBV infection [2–4]. Accumulating data demonstrated that different outcomes of HBV infection primarily were associated with the intensity of anti-viral immune responses [4,5]. In acute hepatitis B (AHB) infection, the innate immune cells occurred rapidly in early stage, and then the adaptive immune cells eliminated HBV completely resulting in a self-limiting hepatitis in adult AHB patients [6]. However, during chronic hepatitis B (CHB) infection, impaired innate and adaptive immune cells were incapable of controlling viral replication and led to disease progression [7–10]. Thus, dissecting the immune responses during early stages of acute HBV infection can provide greater insight into the virus-host interaction and the mechanisms of action on the control of virus. It was well established that innate immune cells including natural killer (NK) cells, NKT cells, and γδ T cells were enriched in liver [11]. Approximately 15%–25% of intrahepatic T cells expressed γδ T cell receptor (TCR) [12], indicating that this specific lymphocyte population might exert important functions in liver diseases. However, little information

Abbreviations: CHB, chronic hepatitis B; AHB, acute hepatitis B; HC, healthy controls; IP-10, interferon inducible protein 10; RANTES, regulated upon activation normal T cell expressed and secreted; HBV, hepatitis B virus; TCR, T cell receptor; NKG2D, natural killer group 2D; TLR, Toll like receptor; IFN, interferon; TNF, tumor necrosis factor; HCV, hepatitis C Virus; ALT, alanine aminotransferase; Ig, immunoglobulin; ACLF, acute on chronic HBV liver failure; CXCR, CXC receptor; HLA, human leukocyte antigen; Gr, Granzyme; CHC, chronic hepatitis C

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Table 1
Clinical data for the enrolled subjects.

Group	Case	Age (Years)	Sex (M:F)	ALT (U/L)	DNA (IU/mL)	sAg/sAb/eAg/eAb/cAb
HC	30	32 (18–48)	19:11	NA	NA	
CHB	28	30 (18–50)	19:9	472 (80–1537)	5.28×10^6 (2.90×10^4–1.57×10^8)	
AHB	29	31 (17–50)	20:9	1003 (220–2299)	1.07×10^5 (1×10^2–3×10^6)	
AHB	1	47	F	1945	5.20×10^2	+/−/+/+/+
AHB	2	25	F	2058	3.90×10^4	+/−/+/+/+
AHB	3	38	M	281	5.27×10^2	+/−/−/+/+
AHB	4	43	M	932	3.09×10^4	+/−/+/−/+
AHB	5	35	M	860	5.00×10^2	+/+/+/−/+
AHB	6	17	M	1115	4.97×10^2	+/−/+/+/+
AHB	7	24	F	1379	1.10×10^4	+/−/+/−/+
AHB	8	19	M	1131	8.16×10^4	+/−/+/−/+
AHB	9	50	F	220	5.00×10^2	+/−/−/−/+
AHB	10	23	M	1727	1.00×10^3	+/−/−/+/+
AHB	11	31	M	2256	6.10×10^3	+/−/−/+/+
AHB	12	23	F	830	1.05×10^3	+/−/−/+/+
AHB	13	18	M	1130	3.00×10^2	+/+/−/+/+
AHB	14	22	M	240	2.00×10^3	+/−/−/+/+
AHB	15	24	M	1137	5.00×10^2	+/−/+/−/+
AHB	16	49	F	831	1.87×10^4	+/−/+/+/+
AHB	17	26	M	989	5.07×10^2	+/−/−/+/+
AHB	18	44	M	465	1.00×10^2	+/−/−/+/+
AHB	19	41	M	294	3.50×10^3	+/−/−/+/+
AHB	20	21	F	221	3.03×10^3	+/−/−/−/+
AHB	21	19	M	2299	4.00×10^2	+/−/+/−/+
AHB	22	35	M	386	3.00×10^6	+/+/−/+/+
AHB	23	21	M	922	5.02×10^2	+/−/−/+/+
AHB	24	35	M	2142	1.44×10^3	+/−/−/+/+
AHB	26	25	F	564	1.00×10^2	+/+/−/+/+
AHB	25	39	F	791	8.62×10^3	+/−/−/−/+
AHB	27	32	M	332	4.95×10^2	+/−/−/+/+
AHB	28	44	M	490	1.02×10^3	+/−/−/+/+
AHB	29	19	M	1132	5.00×10^2	+/−/−/+/+

10 HBeAg-positive and 19 HBeAg-negative AHB patients were enrolled in our study.
28 CHB HBeAg-positive patients were enrolled in this study.
ALT: alanine aminotransferase; s, surface; c, core; Ag, antigen; Ab, antibody; NA, not applicable.
HC, healthy control; CHB, chronic hepatitis B; AHB, acute hepatitis B; M, male; F, female.

is known regarding the function of γδ T cells during early stages of HBV infection.

γδ T cells, which are described to link the innate and adaptive immunity, play critical roles against pathogen infection [13,14]. Based on TCR δ chain expression, those cells can be divided into Vδ1, Vδ2 and Vδ3 T cells in humans. They could directly or specifically recognize pathogen infected cells through different mechanisms. In the early stage of infection, γδ T cells can directly sense the signals from pathogen infected cells through natural killer group 2D (NKG2D), Toll like receptor (TLR) or dectin1, which enable them to respond rapidly [[13,15]]. Moreover, γδ T cells specifically recognize target cells via TCR and induce death-receptor mediated apoptosis, release of cytolytic granules and immune modulatory cytokines including Interferon (IFN)-γ, Interleukin (IL)-17, and tumor necrosis factor (TNF)-α [16–18]. Those γδ T cells with programmed effector functions also promote the early recruitment and activation of neutrophils, macrophage and the boosts of immature dendritic cells and T cells response [13,19].

Indeed, through cytotoxicity and immunomodulatory cytokines mentioned above, γδ T cells are crucial in achieving the initial control of viral infections [20,21]. However, these cells play different roles in regulating the immune response against hepatic viral infections. On the one hand, phosphorylated antigen or heat shock protein activated γδ T cells and their subsets could efficiently clear hepatic viral infections as well as restrict hepatic inflammation and fibrosis in Hepatitis C Virus (HCV) infection patients or animal model [22–24]. On the other hand, however, it was found that γδ T cells lost their capacity to inhibit HBV replication and limit hepatitis-B-e antigen seroconversion in CHB patients by suppressing $CD8^+$ T cell responses [25,26]. Similar effects exerted by γδ T cells were observed in immunotolerance HBV mouse model [9]. Generally, γδ T cells participate in the pathogenesis of hepatic viral infections. Thus the exploration for the distinctive function of γδ T cell in AHB and CHB patients might help reveal the underlying mechanism after HBV infection. However, there are limited data available regarding clinical significance and immune status of these cells in AHB patients with longitudinal follow-up.

By focusing on a cohort of HBV-infected patients, we investigated different function of γδ T cells during acute and chronic HBV infection. Our data showed that decreased peripheral γδ T cells and increased hepatic γδ T cells were found in AHB patients, compared with CHB patients. Moreover, activated γδ T cells exhibited enhanced cytotoxicity and capacity for viral clearance during early stages of AHB infection, indicating their potential role in the control of viral infection. Our present study may be useful for analyzing the function of γδ T cells during HBV infection.

2. Materials and methods

2.1. Subjects

Blood samples were collected from 28 CHB, 29 AHB patients, and 30 age- and sex-matched healthy individuals as controls (HC) (Table 1). All enrolled patients were diagnosed by the criteria described previously [25,27]. AHB patients refer to untreated subjects with elevated serum alanine-aminotransferase (ALT) levels > 100 IU/L, first detection of serum hepatitis-B-surface-antigen and immunoglobulin (Ig) M against hepatitis-B-core antigen. Patients with acute-on-chronic HBV liver failure (ACLF) infection, other virus infection, as well as individuals with autoimmune liver diseases, alcoholic liver disease, and those who met clinical or biological criteria of bacterial or fungal infections, were excluded. CHB patients were untreated hepatitis-B-e-antigen positive

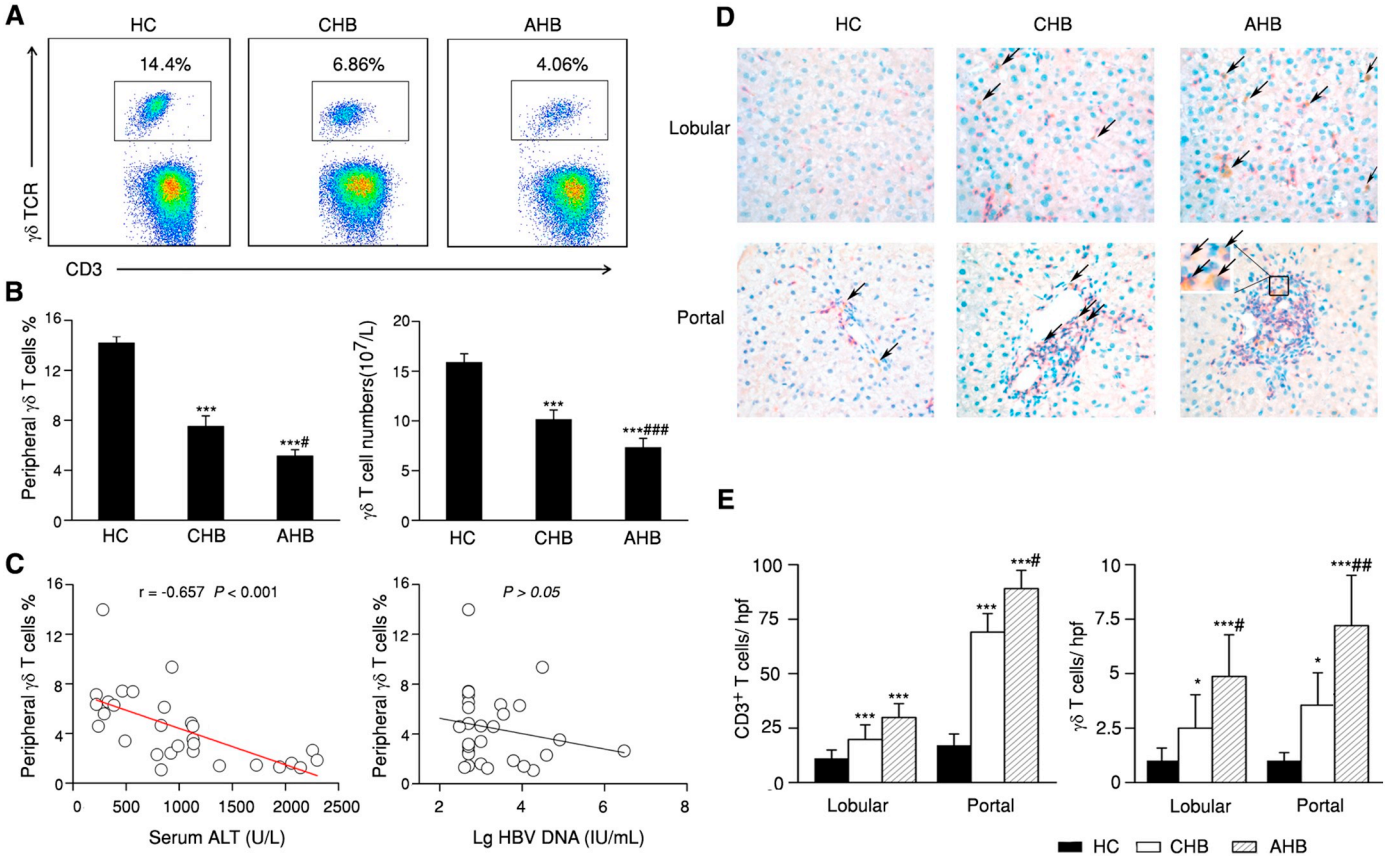

Fig. 1. Quantitative changes of γδ T cells in AHB patients are associated with liver injury. A, Representative dot plots of peripheral γδ T cells in HCs, CHB and AHB patients (gated on $CD3^+$ T cells). B, Statistical analysis of the frequencies and absolute numbers of peripheral γδ T cells in CHB ($n = 28$), AHB patients ($n = 29$) and sex- and age-matched HCs ($n = 30$). * indicates significance when compared with HCs, # indicates significance when compared with CHB patients. C, A correlation analysis between the percentages of γδ T cells, serum alanine aminotransferase (ALT) levels ($r = -0.657$; $P < .001$) and hepatitis B virus (HBV) DNA levels ($P > .05$) in AHB patients. Solid line, linear growth trend. D, Immunohistochemical detection of γδ T cells in the liver tissues of HCs ($n = 5$), CHB ($n = 8$) and AHB patients ($n = 10$) (magnification, 400×). Positively stained cells appeared red (CD3 TCR) and brown (γδ TCR, indicated by arrowheads) are present in both the portal and lobular areas in the livers. The blue color represents the nucleus of the cells. As more hepatic γδ T cells were found in portal of AHB patients, we only amplified a small area and indicated γδ TCR positive cells with arrowheads. E, Pooled data of CD3 and γδ T cell counts in both the portal and lobular areas in HCs ($n = 5$), CHB ($n = 8$) and AHB patients ($n = 10$). All data are presented as the means ± standard errors of the mean and gated on $CD3^+$ T cells. * indicates significance when compared with HCs. # indicates significance when compared with CHB group. * $P < .05$, *** $P < .001$, # $P < .05$, ## $P < .01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CHB patients with ALT > 80 IU/L, HBV DNA > 2×10^4 IU/mL. The study protocol was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Beijing 302 Hospital. Written informed consent was obtained from each subject.

Liver biopsies were collected from 8 CHB and 10 AHB patients. Five healthy tissue samples were also obtained from healthy donors during liver transplantation surgery. For pathological evaluation, liver biopsy specimens were embedded in Tissue Tek for in situ immunohistochemical staining. For hepatic chemokine examination, total RNA from the liver biopsy was isolated for real-time polymerase-chain-reaction.

2.2. Chemotaxis assay

γδ T cells were purified by positive selection using anti-γδ TCR microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). The purity of γδ T cells was > 95%. Cells were loaded to the upper chamber of chemotaxis chamber (Corning, New York, USA) at density of 2×10^6 cells/mL. Medium containing interferon-inducible-protein 10 (IP-10) (R&D, San Diego, USA, 500 ng/mL), or with regulated-upon-activation normal-T-cell expressed and secreted (RANTES) (R&D, San Diego, USA, 100 ng/mL) was loaded into the lower chambers. The chambers were separated by a polycarbonate membrane. After 6 h of incubation, γδ T cells in the lower chamber were enumerated. Chemotaxis Index = migrated γδ T cells/spontaneously migrated γδ T cells. Each experimental condition was performed in -triplicates.

2.3. Viral clearance assay

Purified γδ T cells from AHB patients were pre-treated with anti-γδ TCR antibody (1 μg/mL, coated on 96-well plate) for 3 days and then co-cultured with HepG2.2.15 at different ratios in the presence of IL-2 (100 IU/mL) for 3 days. Supernatants were collected and examined for the HBV DNA load. For transwell and neutralizing experiments, γδ T cells were cultured in the upper chamber and separated from HepG2.2.15 (E:T = 10:1) by transwell membrane. For IFN-γ and TNF-α neutralizing assay, anti-IFN-γ neutralizing antibody (8 μg/mL, R&D, San Diego, USA) or isotype IgG2A antibody (8 μg/mL, R&D, San Diego, USA), as well as anti-TNF-α neutralizing antibody (6 μg/mL, R&D, San Diego, USA) or isotype IgG1 antibody (6 μg/mL, R&D, San Diego, USA) were added to coculture system. The HBV DNA load in culture supernatants were examined 3 days later.

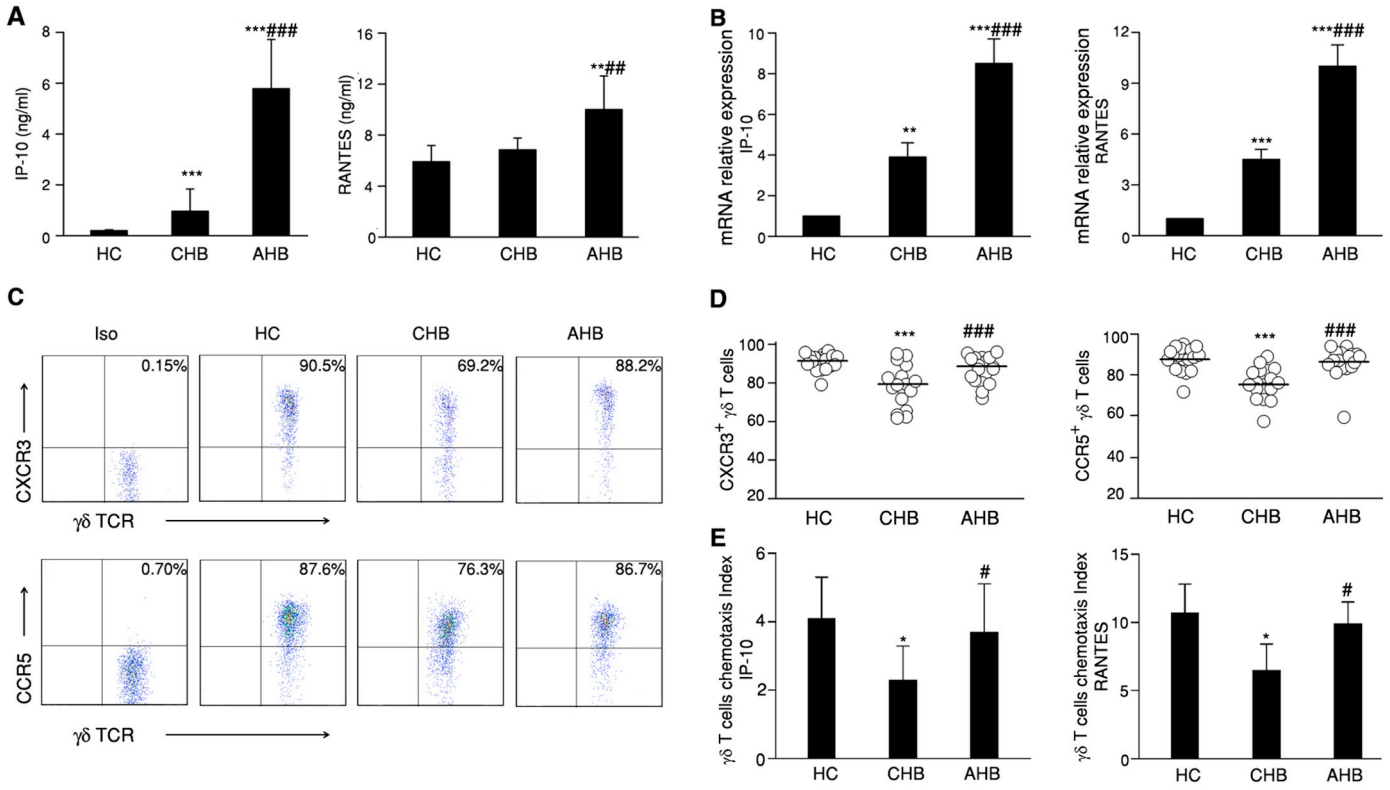

Fig. 2. Increased chemokines might contribute to the increased number of hepatic γδ T cells. A, Statistical data regarding the serum levels of interferon inducible protein 10 (IP-10) and regulated upon activation normal T cell expressed and secreted (RANTES) in HC (n = 30), CHB (n = 28) and AHB (n = 29) groups as quantified by enzyme-linked immunosorbent assay. B, Liver biopsy RNA levels of IP-10 and RANTES in HCs (n = 5), CHB (n = 8) and AHB (n = 10) groups are quantified by real-time PCR. C, Representative dot plots of CXC receptor (CXCR) 3 and CC receptor (CCR) 5 expressed on γδ T cells in three groups. D, Pooled data showing the expression of CXCR3 and CCR5 on γδ T cells in HCs (n = 30), CHB (n = 28) and AHB (n = 29) groups. E, Pooled data showing that γδ T cells from CHB patients (n = 20) exhibit a lower chemotaxis index than those from HCs (n = 20) and AHB (n = 20) group when treated with IP-10 (500 ng/mL) and RANTES (100 ng/mL). All data are presented as the means ± standard errors of the mean. * indicates significance when compared with the HCs. # indicates significance when compared with CHB group. *P < .05, **P < .01, ***P < .001, #P < .05, ##P < .01, ###P < .001.

2.4. Statistical analysis

All data were analyzed using SPSS software (Chicago, IL, USA) and presented as the mean and standard errors of the mean. Multiple comparisons were made between different groups using a Kruskal-Wallis H nonparametric test. Correlation analysis was evaluated by the Spearman rank correlation test. Comparisons between various individuals were performed using a Mann-Whitney U test. Comparisons between different phases of one person were performed using a Wilcoxon matched pairs t-test. For all tests, a two-sided P-value < .05 was considered to be statistically significant.

Other methods and materials (e.g., Flow cytometry and cytolytic killing assay) were presented in supplementary method.

3. Results

3.1. Increased hepatic γδ T cells and decreased peripheral γδ T cells were found in AHB patients

The levels of γδ T cells were assayed in HC, CHB and AHB groups. Flow cytometry results of peripheral γδ T cells (gated on $CD3^+$ T cells) were determined in three groups (Fig. 1A). Notably, the percentage of peripheral γδ T cells was lower in AHB and CHB patients than that in HCs. Statistical data revealed that compared with HCs, the percentage of peripheral γδ T cells significantly decreased in CHB patients and further reduced in AHB patients (Fig. 1B). To validate these findings, absolute numbers of peripheral γδ T cells in three groups were compared. The lowest numbers of peripheral γδ T cells were found in AHB patients compared to HC and CHB groups (Fig. 1B). Furthermore, a significant negative correlation was observed between frequency of peripheral γδ T cells and the serum ALT levels (r = −0.657, P < .001) but not the serum HBV DNA loads (r = −0.130, P > .05; Fig. 1C) in AHB patients. We next examined the distribution of hepatic γδ T cells by immunohistochemical staining (γδ TCR, brown; CD3 TCR, red; nucleus, blue). Compared with HCs, the number of hepatic γδ T cells increased in CHB patients and further increased in AHB patients (Fig. 1D, arrowheads indicated γδ TCR positive cells, we amplified a small portal area of AHB patients and indicated γδ T cells with arrowheads). Statistical analysis of hepatic $CD3^+$ T cells and γδ T cell counts in portal and lobular areas further confirmed this observation (Fig. 1E). Notably, more hepatic γδ T cell were found in inflamed lobular areas of liver in AHB patients. These data manifested the quantitative change of peripheral and hepatic γδ T cells in AHB patients.

3.2. Increased chemokine-induced infiltration of γδ T cells into liver of AHB patients

As quantity of hepatic γδ T cells increased in AHB patients, we further investigated the mechanisms underlying the migration of these cells. Compared with HC and CHB groups, significantly higher levels of IP-10 and RANTES were observed in the sera and liver biopsies of AHB patients (Fig. 2A and B). The corresponding receptors for both IP-10 and RANTES on γδ T cells were screened in three groups. We found that compared with HCs, both CXCR3 (IP-10 ligand) and CCR5 (RANTES ligand) were significantly down-regulated on γδ T cells in CHB but not in AHB patients (Fig. 2C). Statistical analysis further confirmed this

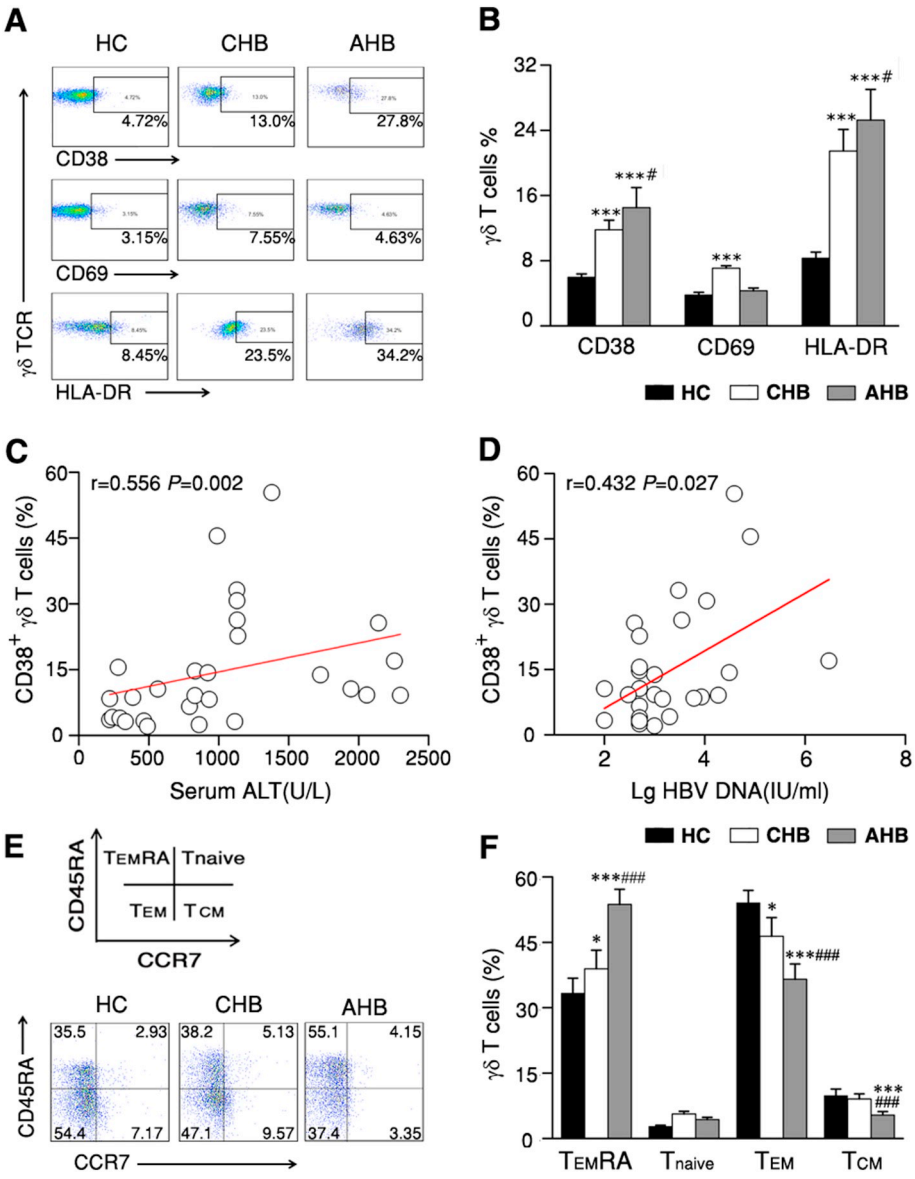

Fig. 3. γδ T cells exhibit increased activated $T_{EM}RA$ phenotype in AHB patients. A, Representative dot plots depict the expression of the activation markers CD38, CD69, and HLA-DR on γδ T cells among HCs, CHB and AHB patients (gated on γδ T cells). B, Pooled data showing the percentages of CD38, CD69 and HLA-DR expression on γδ T cells from HCs (n = 30), CHB (n = 28) and AHB (n = 29) groups. (C,D), Correlation analysis between $CD38^+$ γδ T cells and serum ALT levels (r = 0.556; P = .002), HBV DNA levels (r = 0.432; P = .027) in AHB patients (n = 29). E, Distribution of memory γδ T cell subsets in representative individuals from each group. The values in each quadrant indicate the percentage of each memory subset. Gated on γδ T cells. (T_{CM}: central memory T cells; T_{EM}: effector memory T cells; $T_{EM}RA$: terminally differentiated effector T cells) F, Pooled data of memory γδ T cell subsets in HCs (n = 25), CHB (n = 25) and AHB (n = 25) patients. All data are presented as the means ± standard errors of the mean and gated on γδ T cells. * indicates significance when compared with the HCs. # indicates significance when compared with CHB group. *P < .05, ***P < .001, #P < .05, ###P < .001.

observation (Fig. 2D). Moreover, γδ T cells from CHB patients exhibited reduced chemotaxis compared to those from HC and AHB groups in response to IP-10 and RANTES in vitro (Fig. 2E). These data suggested that maintained chemotaxis capacity and increased IP-10 and RANTES levels promoted the accumulation of γδ T cells into livers of AHB patients.

3.3. γδ T cells from AHB patients exhibited an activated memory $T_{EM}RA$ phenotype

The expression of CD38, CD69, and human leukocyte antigen (HLA)-DR were analyzed to assess the activation status of γδ T cells in three groups (Fig. 3A). Statistical analysis revealed that peripheral γδ T cells derived from AHB patients expressed significantly higher frequencies of CD38 and HLA-DR than HC and CHB groups, while a higher level of CD69 expression on γδ T cells was identified in CHB patients (Fig. 3B). A similar pattern was observed in the MFI of CD38 and HLA-DR expression on γδ T cells in three groups. However, mean fluorescence intensity of CD69 expression on γδ T cells in AHB and CHB patients was higher than HCs (Supplementary Fig. 1). Moreover, the frequency of peripheral $CD38^+$γδ T cells was positively correlated with serum ALT levels (r = 0.556, P = .002; Fig. 3C) and HBV DNA loads (r = 0.432, P = .027; Fig. 3D). CCR7 and CD45RA were used to evaluate memory subsets of γδ T cells in three groups (Fig. 3E). It was revealed that peripheral γδ T cells from AHB patients retained the highest percentage of $CD45RA^+CCR7^-$ $T_{EM}RA$ cells and the lowest percentage of $CD45RA^-CCR7^+$ T_{CM} and $CD45RA^-CCR7^-$ T_{EM} cells among three groups (Fig. 3F). These data suggested that peripheral γδ T cells from AHB patients exhibited an activated memory $T_{EM}RA$ phenotype and that these activated γδ T cells were associated with liver injury and viral loads.

3.4. Cytolytic function of γδ T cells was selectively increased in AHB patients

The expressions of the markers of cytotoxicity: Granzyme A (GrA), Granzyme B (GrB), and perforin in peripheral γδ T cells were firstly assesed in three groups (Fig. 4A). Statistical data indicated that γδ T cells from AHB patients produced greater amount of GrB but reduced GrA compared with HC and CHB groups. Moreover, the frequencies of $perforin^+$γδ T cells showed no significant differences in three groups (Fig. 4B). We further found that the frequency of $CD56^+$γδ T cells in CHB and AHB patients was higher than in HCs. Moreover, the frequency of $NKG2A^+$γδ T cells was higher in CHB patients compared with HCs

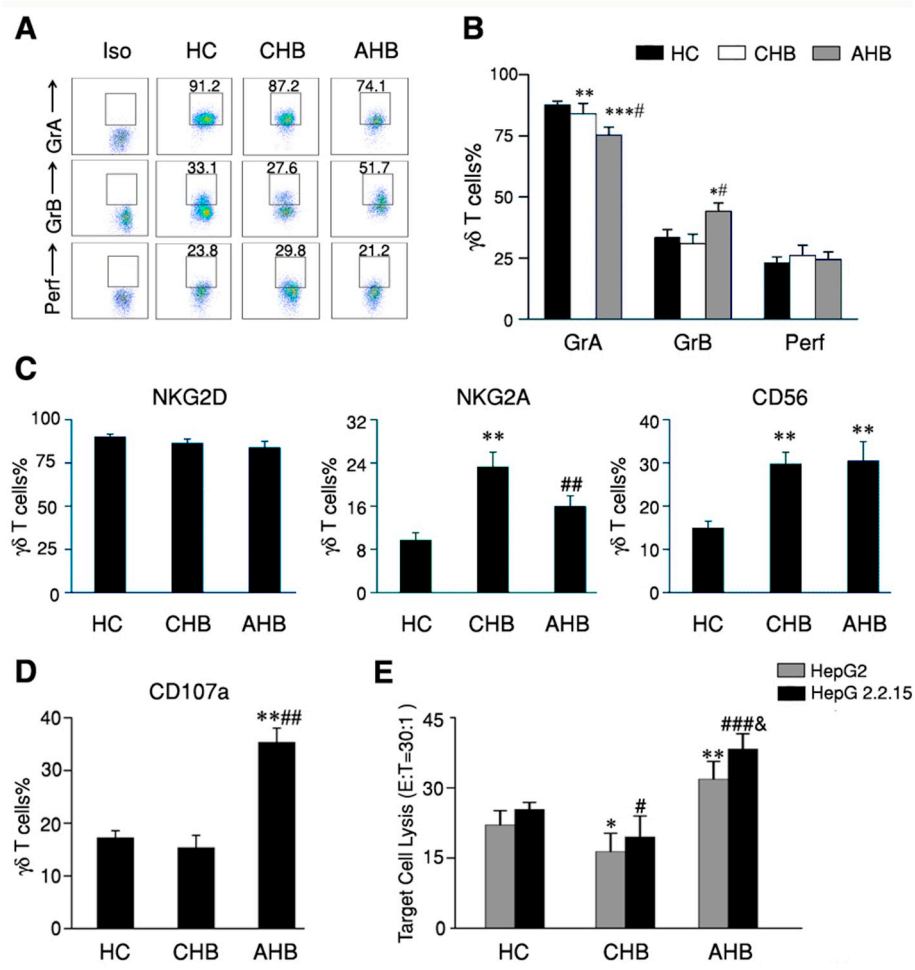

Fig. 4. Enhanced cytolytic capability of γδ T cells in AHB patients. A, Representative dot plots depict the expression of isotype control, granzyme A (GrA), granzyme B (GrB), and perforin (Perf) on γδ T cells from three groups. (Gated on γδ T cells). B, Pooled data of γδ T cells expressing GrA, GrB, and Perf in HCs (n = 25), CHB (n = 20) and AHB (n = 20) patients. * indicates significance when compared with HCs. # indicates significance when compared with CHB group. (**C**) Statistic data regarding the expression of NKG2D, NKG2A, and CD56 on γδ T cells from HCs (n = 25), CHB (n = 20) and AHB (n = 20) patients. * indicates significance when compared with HCs. # indicates significance when compared with CHB group. (**D**) Frequency of γδ T cells expresses CD107a in HCs (n = 25), CHB (n = 20) and AHB (n = 20) patients. * indicates significance when compared with HCs. # indicates significance when compared with CHB group. (**E**) Pooled data show the percentages of HepG2.2.15 cell lysis by γδ T cells from HCs (n = 25), CHB (n = 20), and AHB (n = 20) patients at the E:T ratio of 30:1 compared to the HepG2 control. * indicates significance when compared with percentage of HepG2 lysised by HCs, # indicates significance when compared with percentage of HepG2.2.15 lysised by HCs. & indicates significance when compared with the percentage of HepG2 lysised by AHB patients. All data are presented as the means ± standard errors of the mean. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $\#P < .05$, $\#\#P < .01$, $\#\#\#P < .001$, & < 0.05.

and AHB patients, whereas the frequencies of NKG2D^{+} γδ T cells were similar among three groups (Fig. 4C). Notably, CD107a expression on γδ T cells was significantly upregulated in AHB than in the other two groups (Fig. 4D). γδ T cells were further incubated with HepG2 (as control) and HBV transfected cell line, HepG2.2.15. γδ T cells showed enhanced cytotoxicity to target cells in AHB patients than in HCs and CHB groups. Meanwhile, γδ T cells from AHB patients induced significantly higher lysis of HepG2.2.15 than HepG2 cells (Fig. 4E). Taken together, these data suggested an enhanced cytotoxic capability of peripheral γδ T cells in AHB patients.

3.5. γδ T cells displayed enhanced cytokine production and antiviral activity in AHB patients

To examine non-cytolytic antiviral activity of those cells in AHB patients, cytokines production of γδ T cells were analyzed. More IFN-γ and TNF-α were secreted by peripheral γδ T cells in AHB patients than in other two groups (Fig. 5A). Though γδ T cells were reported as the main source of IL-17 and played crucial role in infectious diseases [13], γδ T cells secreted little IL-17 among three groups (Fig. 5B). To better detect the IL-17 production by γδ T cells, PMA and ionomycine were used to measure the enormous release of IL-17 by those cells. Furthermore, we incubated γδ T cells with HepG2.2.15, supernatants were collected to detect the HBV DNA load. Statistic data revealed that in the presence of γδ T cells from AHB patients, supernatants HBV DNA load of HepG2.2.15 were significantly decreased than other groups (Fig. 5C). To explore underlying mechanisms, a transwell co-culture assay was performed. Separation of γδ T cells and HepG2.2.15 with transwell chamber partially increased HBV DNA load in supernatants when compared with co-culture system without transwell chamber (Fig. 5D). Moreover, neutralizing anti-IFN-γ, anti-TNF-α and corresponding isotype antibodies were added into the transwell co-culture system. Both anti-IFN-γ and anti-TNF-α antibodies significantly increased the HBV DNA loads in supernatant when compared with isotype antibodies (Fig. 5D). Briefly, peripheral γδ T cells from AHB patients exhibited enhanced cytolytic and antiviral activity at least partially via secretion of IFN-γ and TNF-α.

3.6. Longitudinal analysis of the phenotypes of γδ T cell in AHB patients

The serum ALT, HBV DNA and phenotypes of γδ T cells were longitudinally analyzed in peripheral blood of AHB patients during the acute (median: 2 weeks since the clinical onset; range: 1–3 weeks) and convalescence phases (median: 16 weeks since the clinical onset; range: 8–28 weeks) for 8 AHB patients (Fig. 6A). It was found that frequency of peripheral γδ T cells was significantly increased with reducing of serum HBV DNA and ALT levels to normal levels from the acute phase to convalescence phase in AHB patients (Fig. 6B). Remarkably, the expressions of CD38, HLA-DR, and T_{EM}RA on γδ T cells were all found to be significantly reduced, while T_{EM} γδ T cells significantly increased during convalescence phase compared to acute phase in the same AHB patient (Fig. 6C). Meanwhile, cytotoxicity markers (GrB, CD56, CD107a) and IFN-γ were significantly decreased in γδ T cells during convalescence phase (Fig. 6D). These data demonstrated that the status of activation, cytotoxic and viral clearance capability of γδ T cells was decreased during the recovery of AHB patients.

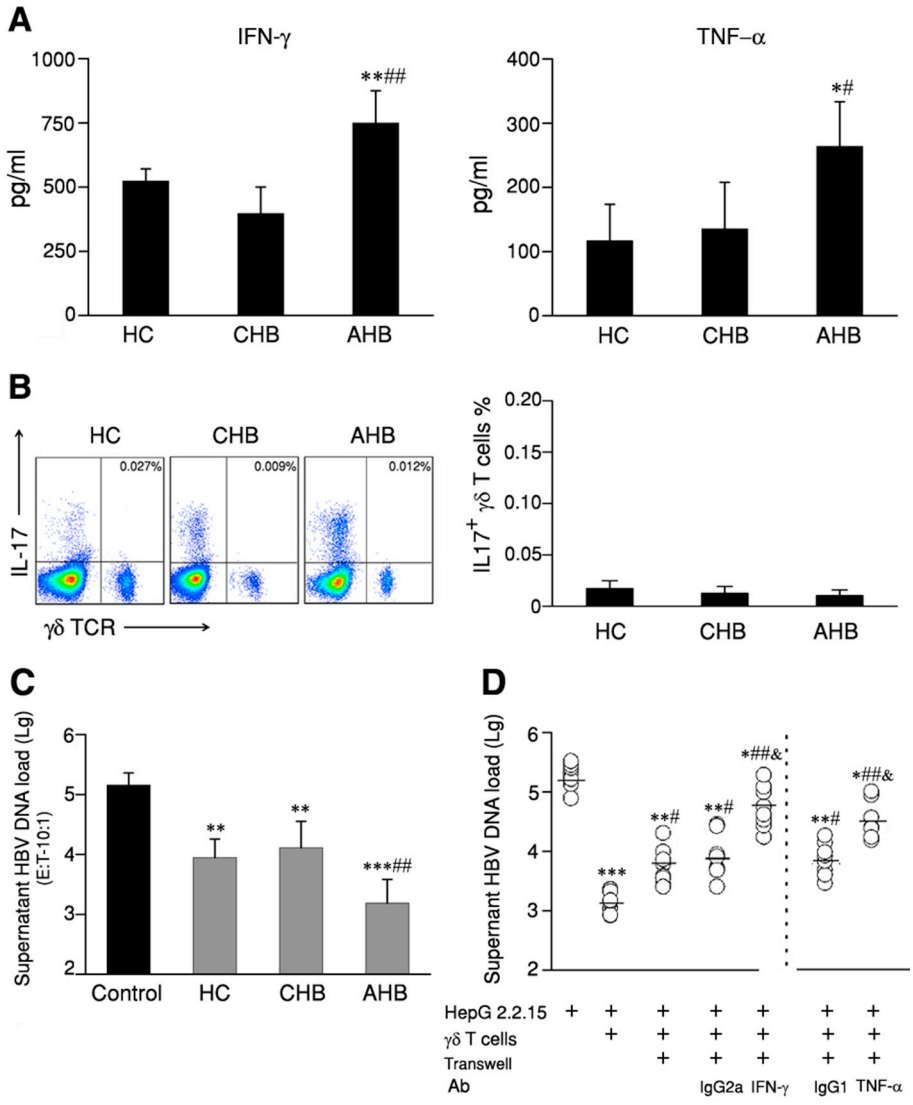

Fig. 5. γδ T cells from AHB patients exhibit increased cytokine production and viral clearance capacity. A, Quantitative analysis of IFN-γ and TNF-α produced by γδ T cells from HCs (n = 20), CHB (n = 20) and AHB (n = 20) patients. B, Representative dot plots show the secretion of IL-17 on γδ T cells from three groups (Gated on $CD3^+$ T cell, left). Statistic data indicating the percentage of IL-17 secreted by γδ T cells from HCs (n = 15), CHB (n = 15) and AHB (n = 15) patients (Gated on $CD3^+$ T cell, right). C, Pooled data show the HBV DNA load in the supernatants of the coculture system: γδ T cells from HCs (n = 10), CHB patients (n = 10) and AHB patients (n = 10) incubated with HepG2.2.15 cells (E:T = 10:1). HepG2.2.15 only was considered as control. * indicates significance when compared with HepG2.2.15 control. # indicates significance when compared with HCs. D, Both the transwell system and neutralization assay for IFN-γ and TNF-α in a coculture system (γδ T cells:HepG2.2.15 = 10:1) could partially restore the HBV DNA load in the supernatants. All data are presented as the means ± standard errors of the mean. * indicates significance when compared with HepG2.2.15 only group. # indicates significance when compared with the γδ T cells and HepG2.2.15 coculture system. & indicates significance when compared with the transwell coculture system. *P < .05, **P < .01, ***P < .001, #P < .05, ##P < .01, & P < .05.

4. Discussion

Human γδ T cells play critical roles in regulating the immune response against viral infections [22]. However, the specific function of these cells during early acute HBV infection remains largely unknown. In the present study, decreased peripheral and increased hepatic γδ T cells were found in AHB patients. Accumulation of hepatic γδ T cells in AHB patients was attributed to the chemoattractive/chemotactic effects elicited by IP-10 and RANTES. Unlike that from HCs and CHB patients, γδ T cells from AHB patients were more activated and exhibited elevated cytotoxicity and antiviral activity, which were further confirmed in a longitudinal study.

Increasing evidence revealed that adequate innate immune response is crucial for achieving early effective viral control [6]. Our previous study demonstrated that impaired Vδ2 γδ T cells contributed to HBV progression in CHB patients [25]. In order to reveal role of γδ T cells in early HBV infection, we explored for the characteristics of those cells in AHB patients. Firstly, similar to the studies in chronic HCV infection and ACLF [28,29], decreased peripheral γδ T cells were correlated with liver injury and increased hepatic γδ T cells were found in AHB patients compared with HCs and CHB patients. Particularly, more hepatic γδ T cells were detected in portal areas than lobular areas in AHB patients. As the recruitment of immune cells into hepatic portal areas were often related to virus clearance or liver damage [6,10], the increased number of hepatic γδ T cells might imply a pathogenic role during acute HBV infection. Secondly, we further investigated the cause of increased hepatic γδ T cell infiltration in AHB patients. Chemokines which were associated with migration of γδ T cells [25,30] were analyzed. IP-10 and RANTES, but not monocyte chemotactic protein 1 or stromal cell derived factor 1α (data not shown), were significantly increased in serum and liver biopsies of AHB patients than the other two groups. Unlike attenuated chemotactic capability of γδ T cells in CHB patients [25], these cells from AHB patients maintained a complete chemotactic capacity as HCs. Thirdly, more effector ($T_{EM}RA$) γδ T cells which was reported to have regulatory and virus clearance capacity [13] were found in AHB patients. More activated γδ T cells were found in AHB patients and dominated by activation receptor CD38 and HLA-DR. The activated $CD38^+$ γδ T cells were significantly positively correlated with serum ALT and HBV DNA load in AHB patients. As serum ALT was a clinical parameter of liver injury in HBV infection patients, the activated γδ T cells could be associated with liver injury. These findings explain status changes of γδ T cells in AHB patients, which indicate the participation of these cells in acute HBV pathogenesis.

During acute HBV infection, the early stages are characterized by temporary cytotoxicity and viral clearance function of innate immune cells [6], antiviral functions of γδ T cells in AHB patients were examined in two aspects: cytolytic capacity and non-cytolytic (cytokine production) capability. We firstly analyzed cytolytic potential of γδ T

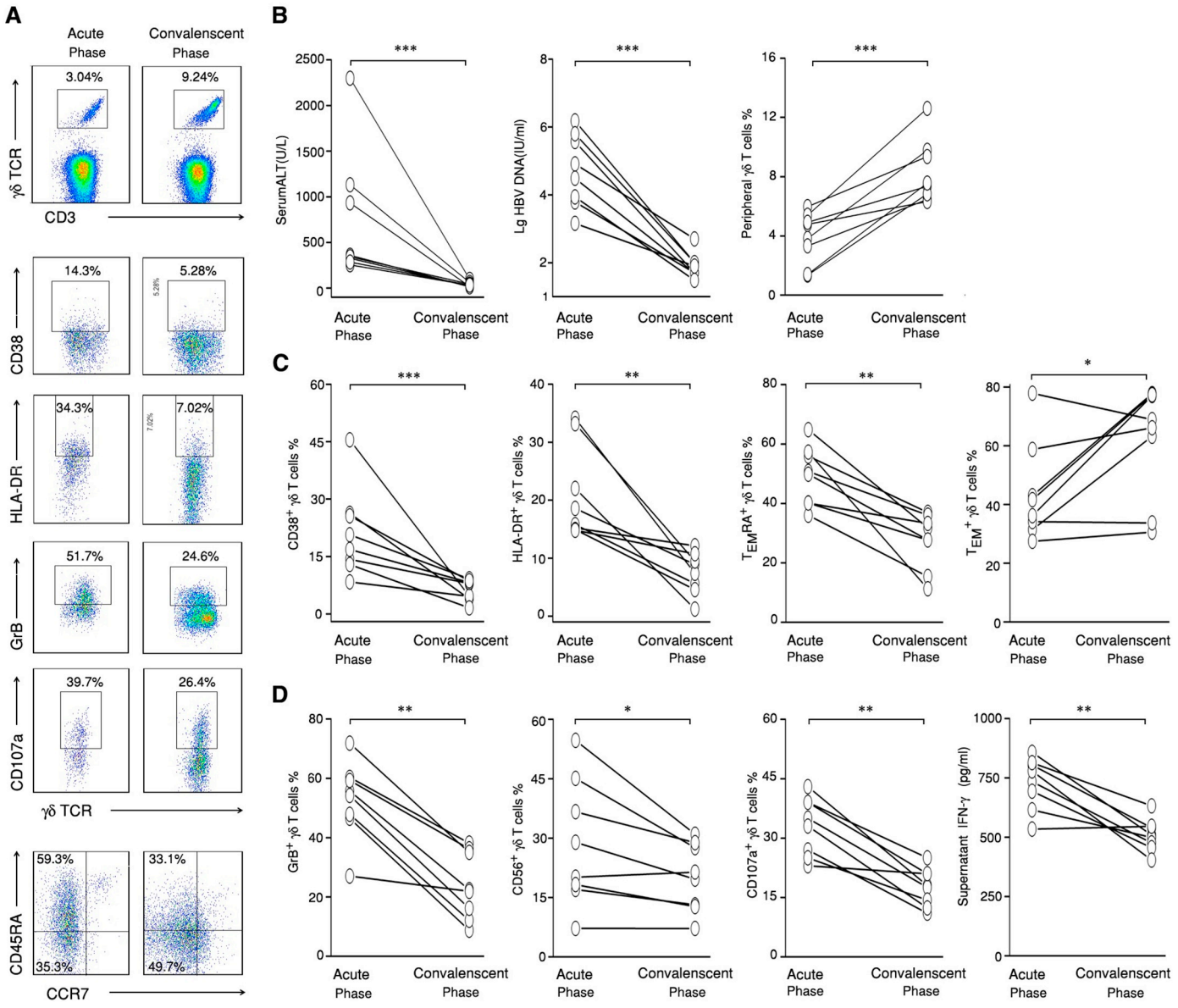

Fig. 6. Dynamic expression of the clinical parameters and immunological status of γδ T cells in AHB patients. A, Representative dot plots indicate the γδ T cell frequencies and immune status expression markers on γδ T cells in AHB patients during the acute and convalescent phases of infection. Values indicate the percentages of immune marker positive γδ T cells. B, The dynamic changes in the serum ALT levels, HBV DNA, and the percentages of peripheral γδ T cells were analyzed in AHB patients during the acute and convalescent phases. C, CD38, HLA-DR and memory phenotype expression of γδ T cells were detected in the same AHB patients during the acute and convalescent phases. D, GrB, CD56 and CD107a expression on γδ T cells from AHB patients, as well as IFN-γ production were probed during the acute and convalescent phases. Each plot represents one patient. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

cells in three groups. It was reported that γδ T cells displayed impaired [31] or enhanced [29] cytolytic activity in CHB or ACLF patients, respectively. As degranulation molecules and NK cell marker were considered to be features of activated innate immune cells during viral clearance [32], increased granzyme B, CD56 and CD107a and lower expression of negative regulated marker NKG2A [6] on γδ T cells indicated elevated cytotoxicity of those cells in AHB patients. Furthermore, we explored the cytolytic capability of γδ T cells by incubating them with HBV transfected cell line HepG2.2.15 and control (HepG2). Unlike lower cytolytic capacity of these cells in CHB and chronic hepatitis C (CHC) patients [31,32]., γδ T cells from AHB patients induced more lysis of HepG2.2.15 than other two groups. Intriguingly, γδ T cells from AHB patient lyzed more HepG2.2.15 than HepG2 control which was attributed to the HBV antigens or HSP expression in HepG2.2.15 that could activate γδ T cells. Subsequently, non-cytolytic antiviral mechanisms of those cells were explored. Compared with CHB patients and HCs, elevated IFN-γ and TNF-α but not IL-17 production of γδ T cells were found in AHB patients. Interestingly, as the main source of IL-17 in mice model [14], γδ T cells barely secret IL-17 in three groups. This may be attributed to the different subjects we studied. Meantime, situations were different in ACLF patients where γδ T cells secreted increased TNF-α and IL-17 [29], which may ascribed to the different immune status of patients and different stimulated condition. We further proved that γδ T cells from AHB patients displayed enhanced inhibition of HBV replication in HepG2.2.15. Then, transwell assays and neutralizing assays were performed to detect the underlying mechanisms. Like CHC patients [22], γδ T cells from AHB patients significantly inhibited HBV replication at least partially through IFN-γ and TNF-α production. As decreased secretion of IFN-γ by γδ T cell may lead to viral persistence in CHB patients [[31]], we concluded that sufficient cytokine production by γδ T cells were important for achieving viral clearance during acute HBV infection. Finally, using longitudinal

follow-up study, we further demonstrated that activated functional γδ T cells possessed enhanced cytotoxic and antiviral capability. During convalescence phase, the frequency of peripheral γδ T cells was increased, cytotoxicity and cytokine (IFN-γ) production of γδ T cells were decreased in AHB patients compared with those observed in acute phase. These results were similar to the decreased cytotoxicity of NK cells and the suppression of activated $CD8^+$ T cells as infection resolved in AHB patients [6]. Collectively, we concluded that adequate cytotoxicity and viral clearance performed by γδ T cells in AHB patients were crucial for achieving effective viral control.

This study had some limitations: firstly, only the function of total γδ T cells but not their subsets (Vδ1, Vδ2 T cells) were analyzed. Secondly, in our longitudinal follow up study, only blood samples of acute and convalescence phase were collected. It should be informative to analyze γδ T cell responses during incubation phase of acute HBV infection in further studies. Thirdly, although intrahepatic distribution of γδ T cells was detected via immunohistochemical staining, our study lacked description of immune status and antiviral activity of these hepatic γδ T cells. It was hard to obtain enough liver specimens from AHB patients to perform the relevant experiments. Thus, further studies should be designed to detect the phenotype and functional changes of hepatic γδ T cells in AHB patients.

In conclusion, this study elucidated the quantity and functional changes of γδ T cells during acute HBV infection. In AHB patients, the activated γδ T cells exhibited increased cytolytic and anti-viral responses during acute phase, which was decreased to normal range during convalescence phase along with the recovery of liver injury and HBV clearance. These findings extend our knowledge regarding the features of γδ T cells in acute HBV infection.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.03.005.

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Disclosure of conflict of interest

All authors declare that they have no conflict of interest.

Author contributions

Zhenghu Jia, Yuanyuan Li and Jingya Wang: acquisition and analysis of data, wrote the main text; Ji-Yuan Zhang and Ang Huang prepared the main figures; Xiaodong Guo, Zhenyu Zhu: prepared the table and collect blood and liver biopsy samples. Fu-Sheng Wang and Xiaoli Wu: designed of the study, modified the manuscript. All authors reviewed the manuscript.

References

[1] B. Rehermann, M. Nascimbeni, Immunology of hepatitis B virus and hepatitis C virus infection, Nat. Rev. Immunol. 5 (2005) 215–229.
[2] F. Stelma, S.B. Willemse, R. Erken, et al., Dynamics of the immune response in acute hepatitis B infection, Open Forum Infect. Dis. (2017), https://doi.org/10.1093/ofid/ofx231.
[3] P. Fisicaro, C. Valdatta, C. Boni, et al., Early kinetics of innate and adaptive immune responses during hepatitis B virus infection, Gut 58 (2009) 974–982.
[4] K. Michitaka, A. Hiraoka, Y. Tokumoto, et al., Clinical features of adult patients with acute hepatitis B virus infection progressing to chronic infection, Int. J. Hepatol. (2014), https://doi.org/10.1155/2014/358206.
[5] B. Rehermann, A. Bertoletti, Immunological aspects of antiviral therapy of chronic hepatitis B virus and hepatitis C virus infections, Hepatology 61 (2015) 712–721.
[6] C. Dunn, D. Peppa, P. Khanna, et al., Temporal analysis of early immune responses in patients with acute hepatitis B virus infection, Gastroenterology 137 (2009) 1289–1300.
[7] H.H. Sun, D.F. Zhou, J.Y. Zhou, The role of DCs in the immunopathogenesis of chronic HBV infection and the methods of inducing DCs maturation, J. Med. Virol. 88 (2016) 13–20.
[8] B. Ye, X. Liu, X. Li, et al., T-cell exhaustion in chronic hepatitis B infection: current knowledge and clinical significance, Cell Death Dis. (2015), https://doi.org/10.1038/cddis. 2015.42.
[9] X. Kong, R. Sun, Y. Chen, et al., Gammadelta T cells drive myeloid-derived suppressor cell-mediated CD8+ T cell exhaustion in hepatitis B virus-induced immunotolerance, J. Immunol. 193 (2014) 1645–1653.
[10] J.Y. Zhang, Z. Zhang, F. Lin, et al., Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B, Hepatology 51 (2010) 81–91.
[11] B. Gao, W.I. Jeong, Z. Tian, Liver: an organ with predominant innate immunity, Hepatology 47 (2008) 729–736.
[12] V. Racanelli, B. Rehermann, The liver as an immunological organ, Hepatology 43 (2006) S54–S62.
[13] M. Bonneville, R.L. O'Brien, W.K. Born, Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity, Nat. Rev. Immunol. 10 (2010) 467–478.
[14] X. Wang, Z.G. Tian, γδ T cells in liver diseases, Front. Med. 12 (2018) 262–268.
[15] C. Khairallah, J. Déchanet-Merville, M. Capone, γδ T cell-mediated immunity to cytomegalovirus infection, Front. Immunol. (2017), https://doi.org/10.3389/fimmu.2017.00105.
[16] H.W. Cho, S.Y. Kim, D.H. Sohn, et al., Triple costimulation via CD80, 4-1BB, and CD83 ligand elicits the long-term growth of Vgamma9Vdelta2 T cells in low levels of IL-2, J. Leukoc. Biol. 99 (2016) 521–529.
[17] D. Kabelitz, J. Dechanet-Merville, Editorial: "recent advances in gamma/Delta T cell biology: new ligands, new functions, and new translational perspectives", Front. Immunol. (2015), https://doi.org/10.3389/fimmu.2015.00371.
[18] N. Schmolka, K. Serre, A.R. Grosso, et al., Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory gammadelta-T cell subsets, Nat. Immunol. 14 (2013) 1093–1100.
[19] L. Zheng, Y. Hu, Y. Wang, et al., Recruitment of neutrophils mediated by Vγ2 gammadelta T cells deteriorates liver fibrosis induced by Schistosoma japonicum infection in C57BL/6 mice, Infect. Immun. (2017), https://doi.org/10.1128/IAI.01020-16.
[20] A.K.L. Cheung, H.Y. Kwok, Y. Huang, et al., Gut-homing Delta42PD1+Vdelta2 T cells promote innate mucosal damage via TLR4 during acute HIV type 1 infection, Nat. Microbiol. 2 (2017) 1389–1402.
[21] Z. Djaoud, L.A. Guethlein, A. Horowitz, et al., Two alternate strategies for innate immunity to Epstein-Barr virus: one using NK cells and the other NK cells and gammadelta T cells, J. Exp. Med. 214 (2017) 1827–1841.
[22] C. Agrati, T. Alonzi, R. De Santis, et al., Activation of Vgamma9Vdelta2 T cells by non-peptidic antigens induces the inhibition of subgenomic HCV replication, Int. Immunol. 18 (2006) 11–18.
[23] Z. Meng, J. Wang, Y. Yuan, et al., Gammadelta T cells are indispensable for interleukin-23 mediated protection against Concanavalin-A-induced hepatitis in hepatitis B virus transgenic mice, Immunology 151 (2017) 43–55.
[24] L. Hammerich, J.M. Bangen, O. Govaere, et al., Chemokine receptor CCR6 dependent accumulation of gammadelta T cells in injured liver restricts hepatic inflammation and fibrosis, Hepatology 59 (2014) 630–642.
[25] X. Wu, J.Y. Zhang, A. Huang, et al., Decreased Vdelta2 gammadelta T cells associated with liver damage by regulation of Th17 response in patients with chronic hepatitis B, J. Infect. Dis. 208 (2013) 1294–1304.
[26] Q. Lai, S. Ma, J. Ge, et al., TCRgammadelta(+)CD4(−)CD8(−) T cells suppress the CD8(+) T-cell response to hepatitis B virus peptides, and are associated with viral control in chronic hepatitis B, PLoS One (2014), https://doi.org/10.1371/journal.pone.0088475.
[27] Chinese Society of Hepatology, Prevention and treatment of viral hepatitis, Chin. J. Inf. Dis. 19 (2001) 56–62.
[28] G. Par, D. Rukavina, E.R. Podack, et al., Decrease in CD3-negative-CD8dim(+) and Vdelta2/Vgamma9-TcR+ peripheral blood lymphocyte counts, low perforin expression and the impairment of natural killer cell activity is associated with chronic hepatitis C virus infection, J. Hepatol. 37 (2002) 514–522.
[29] M. Chen, P. Hu, H. Peng, et al., Enhanced peripheral gammadelta T cells cytotoxicity potential in patients with HBV-associated acute-on-chronic liver failure might contribute to the disease progression, J. Clin. Immunol. 32 (2012) 877–885.
[30] J. Sanchooli, N. Sanadgol, M. Kazemi Arababadi, et al., CCR5 plays important roles in hepatitis B infection, Viral Immunol. 27 (2014) 2–6.
[31] M. Chen, D. Zhang, W. Zhen, et al., Characteristics of circulating T cell receptor gamma-delta T cells from individuals chronically infected with hepatitis B virus (HBV): an association between V(delta)2 subtype and chronic HBV infection, J. Infect. Dis. 198 (2008) 1643–1650.
[32] W. Yin, S. Tong, Q. Zhang, et al., Functional dichotomy of Vdelta2 gammadelta T cells in chronic hepatitis C virus infections: role in cytotoxicity but not for IFN-gamma production, Sci. Rep. (2016), https://doi.org/10.1038/srep26296.