Cytokines elevated in patients with HBV-related acute-on-chronic liver failure promote NK cell mediated cytotoxicity through TRAIL

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Background and aims: The role of NK cells on inducing liver injury in patients with HBV-related acute-on-chronic liver failure (HBV-ACLF) is not well understood. The aim of this study was to determine the cytotoxicity of tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-expressed NK cells from HBV-ACLF patients and facilitate a better understanding of the immune pathogenesis of HBV-ACLF. **Methods:** Peripheral blood samples were obtained from HBV-ACLF patients, mild chronic hepatitis B (CHB) patients and healthy controls (HC). Circulating NK cells phenotype was determined using flow cytomerty. Serum cytokine concentrations were ascertained using the CBA Inflammation kit. Cell apoptosis was analyzed using the FITC-annexin V Apoptosis Detection Kit. **Results:** Peripheral NK cells from HBV-ACLF expressed higher levels of TRAIL than those from CHB and HC. Expression of TRAIL on NK cells were correlated positively with serum IL-6 and IL-8 concentrations in HBV-ACLF patients, which is further confirmed by cytokines stimulation in vitro. NK cells caused a significant increase of apoptotic hepatocytes, and further increased the frequency of apoptosis in IL-6&IL8-stimulated hepatocytes; the apoptosis was then inhibited partially by an anti-TRAIL monoclonal antibody. **Conclusion:** These results suggested that inflammation cytokines elevated in patients with HBV-ACLF may promote NK cell mediated cytotoxicity through TRAIL pathway.

Key words: Hepatitis B virus; tumor necrosis factor-related apoptosis-inducing ligand; Inflammation; apoptosis

1. Introduction

Acute-on-chronic liver failure (ACLF) encompasses patients with previously well-compensated liver disease in whom an acute decompensation of liver function occurs due to a precipitating event [1, 2]. In China, HBV-infected ACLF account for more than 80% of ACLF cases owing to a high incidence of chronic HBV infection [3]. The lack of effective therapeutic options for the patients with ACLF results in a high mortality rate [3, 4].

Although detail mechanisms for liver failure caused by HBV infection are unclear, previous studies have shown that the role of immune response is crucial in HBV-related liver diseases [5-7]. Liver injury in patients with HBV infection has generally been attributed to cytotoxicity of infected hepatocytes by virus-specific $CD8^+$ T cells. However, activated HBV-specific CD8 T cells usually present at high frequencies in the livers of patients controlling HBV infection, while these cells often display functional impairment during chronic hepatitis B virus infection [8-10]. Meanwhile, there also exists a reduction in $CD4^+$ T lymphocytes and suppressive $CD4^+$ Tregs cells, which indicating an adaptive immune dysfunction in HBV-ACLF patients [11]. All of these findings suggest that non-virus specific inflammatory cells may actively participate in HBV-associated liver failure.

Natural killer (NK) cells are abundant in the liver and serve as a major innate immune component against viral infection [12]. Recent studies have suggested that NK cells may contribute to liver pathogenesis in patients with HCV or HBV infection [13, 14]. During HBV infection, increased IFN-α production and cytolytic capacity of NK cells may play a role in HBV control as well as liver injury [15, 16]. Activated NK cells may contribute to hepatocyte necrosis through Fas/FasL and NKG2D/NKG2D ligand pathway in virus-induced liver failure [17]. NK cells can

mediate hepatocyte apoptosis by up-regulating Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in CHB patients with hepatic flares and TRAIL expression on NK cells were up-regulated in immune-activated CHB patients [18], indicating TRAIL-mediated apoptosis may be increased during HBV-ACLF with acute severe liver injury. However, the role of TRAIL-expressed NK cells on inducing liver injury in HBV-ACLF patients is not well understood.

Thus, we will first compare the expression of TRAIL on NK cells in CHB, HBV-ACLF patients and healthy controls, and further analyze the cytotoxicity of TRAIL-expressed NK cells from HBV-ACLF patients to facilitate a better understanding of the immune pathogenesis of HBV-ACLF.

2. Materials and Methods

2.1. Patients

Peripheral blood samples at enrollment were obtained from 40 patients with HBV-ACLF and 40 patients with mild CHB between Jan. 2012 to Jan. 2013 from the Liver Failure Treatment and Research Center of Beijing 302 Hospital. ACLF was diagnosed as following criteria [1]: (1) preexisting chronic liver diseases; (2) acute deterioration with increasing jaundice (serum total bilirubin > 171.0 μmol/L or a daily elevation > 17.1 μmol/L); (3) plasma prothrombin activity (PTA < 40%) or international standard ratio (INR) > 1.5. The criteria for ACLF have been widely used in China and are similar with the consensus suggested by Asian Pacific Association for the Study of the Liver (APASL) [2]. CHB was diagnosed according to the criteria recommended by the Chinese Society of Infectious Diseases, and the Chinese Society of Hepatology [19]. Each patient received comprehensive medical intervention and

antiviral treatment according to the diagnostic and treatment guidelines for liver failure [1].

All patients were negative for antibodies against hepatitis A, hepatitis C, hepatitis D, hepatitis E, and human immunodeficiency viruses, as well as no evidence of autoimmune liver disease, no hepatocellular carcinoma or metastatic liver tumor, and no immunosuppressive medication at the time of the study. Peripheral blood samples from 40 age- and sex-matched healthy volunteers during the same period in the Health Examination Centre of Beijing 302 hospital were included as controls.

The protocol had been approved by the Ethical Committee of Beijing 302 Hospital and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient or next of kin before entering the study protocol.

2.2. Flow cytometric phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from patients with CHB, HBV-ACLF and healthy controls by gradient centrifugation on Ficoll-Hypaque (Sigma-Aldrich, MO, USA). For phenotypic analysis, PBMCs were stained with the conventional color immunofluorescence using PerCP-conjugated anti-CD3, FITC-conjugated anti-CD56, APC-conjugated anti-CD69, and PE-conjugated anti-TRAIL (All from BD Biosciences, San Diego, CA) for 20 min in the dark at room temperature. The cells were then washed once and performed by flow cytometry on a BD FACS Canto‖cytometer (BD Biosciences). The data were analyzed by using FlowJo7.6.1 software (TreeStar Inc., Ashland, OR).

2.3. Determination of serum cytokine concentrations

Serum cytokine concentrations were ascertained using the CBA Inflammation kit (BD Biosciences) according to the manufacturer's protocols. In brief, 50μl of patient serum or standard recombinant protein diluents was added to a mixture of capture beads coated with mAb to a panel of cytokines (IL-8, IL-1β, IL-6, IL-10, TNFα, and IL-12p70) and a PE-conjugated detection reagent. After 3 h incubation, the capture beads were washed and acquired on a FACS Canto‖cytometer (BD Biosciences). Using the recombinant standards and the BDCBA Software, cytokine concentrations were quantified for each sample.

2.4. Cytokine induced NK cells activation and up-regulated TRAIL expression

PBMCs isolated from CHB and ACLF patients were cultured in a medium alone or with IL-6 or IL-8 (Peprotech, Rocky Hill, NJ) at a final concentration of 10ng/ml for 24 h. Cytokine-induced CD69 and TRAIL expression on NK cells were analyzed by flow cytometry as described previously.

2.5. Purification of NK cells

NK cells were purified from fresh isolated PBMCs using NK cell enrichment kit (Stem cell technologies, Vancouver, BC, Canada) as per the manufacturer's instructions. In brief, PBMCs were incubated with a combination of monoclonal antibodies bound in bispecific Tetrameric Antibody Complexes (TAC) which are directed against cell surface antigens on human blood cells (CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA-DR, glycophorin A) and dextran for 10 m. Then magnetic dextran iron particles were added for additional 5 m. NK cells were negative

selected from magnetic microbeads. The purity of NK cells was maintained between 85 and 95% by flow cytometry analysis.

2.6. Apoptosis assay

The human immortal hepatocyte cell line L02 was purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). The cell line was maintained in high-glucose DMEM supplemented with 10% FBS at 37°C in a humidified chamber containing 5% CO2. Purified NK cells from patients were pre-incubated with 10ng/ml IL-6 and IL-8 for 24 h to make high expression of TRAIL. Simultaneously, the L02 cells were trypsinized and plated into a 24-well culture plate at 2×10^5 cells/well with or without 10ng/ml IL6 and IL-8 stimulation for 24 h. NK cells were treated with or without 10ng/ml TRAIL blocking antibody (R&D system, MN, USA) for 2 h, and then added to L02 cells at a ratio of 10:1 for 6 h at 37℃. Apoptosis of L02 cells was analyzed using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to manufacturer's instructions. The degree of activated pancaspases was determined using the CaspACE™ FITC-VAD-FMK kit (Promega, Madison, WI) by flow cytometry according to the manufacturer's instructions.

2.7. Statistical Analysis

The data were expressed as mean ± SD or median and range. All data processing was carried out using SPSS for Windows version 17.0 (SPSS, Chicago, IL). Statistical significance was determined by a two-tailed Student's t test (for continuous variables) or Mann-Whitney U test (for non-normal distribution parameters) as appropriate. Categorical data were compared by the χ^2 test. Spearman's correlation analysis was

used to assess relationships between two parameters. A two-sided *P* value less than 0.05 considered statistically significant.

3. Results

3.1. Characteristics of patients

The baseline characteristics of patients with CHB and HBV-ACLF, as well as healthy controls were summarized in Tab.1. No significant differences were determined among three groups for the age and gender ratio. The patients with HBV-ACLF have more disease severity than those with CHB according to higher levels of serum total bilirubin (TBil) and international normalized ratio (INR).

3.2. Increased CD56^{bright} NK subsets in patients with HBV-related acute-on-chronic liver failure

The frequencies of circulating total NK cells ($CD3^-CD56^+$) and NK subsets ($CD3^-CD56^{bright}$ and $CD3^-CD56^{dim}$) in all enrolled subjects were analyzed by flow cytometry (Fig. 1A). The results revealed that the total NK cell frequencies were significantly decreased in both CHB and HBV-ACLF patients in comparison with those in HC subjects (both $p = 0.001$). However, the frequencies of $CD3^-CD56^{bright}$ NK cells were significantly increased in both CHB and HBV-ACLF patients compared to the HC group (both $p < 0.05$). Meanwhile, HBV-ACLF patients had much higher frequency of $CD3^-CD56^{bright}$ NK cells than CHB patients, indicating a re-distribution in peripheral NK cell component in patients with liver failure.

3.3. NK cells from HBV-ACLF patients express high levels of TRAIL and are highly activated

We then compared the expression of TRAIL in HC, CHB and HBV-ACLF patients (Fig.1B). The percentage of TRAIL expression on NK cells was 12.5 ± 7% in HBV-ACLF patients, which was significant higher than those in HC and CHB patients (both $p < 0.001$). Further analysis showed that the majority of TRAIL was shown to be on the $CD56^{bright}$ subset of NK cells rather than the larger $CD56^{dim}$ subset ($p < 0.001$, Fig.1C).

Next, expression of CD69 was analyzed to assess the activation status of NK cells in HC, CHB and HBV-ACLF patients (Fig.2A). As shown in Fig.2B, average frequency of CD69 expression on NK cells was 24.1% in HBV-ACLF patients, which was significantly increased in comparison with HC (2.2%, $p < 0.001$) and CHB patients (3.1%, $p < 0.001$), indicating that the NK cells in HBV-ACLF patients were highly activated.

3.4. Serum IL-6 and IL-8 concentrations are increased in patients with HBV-ACLF and correlated with TRAIL expression on NK cells

Previous studies have indicated that inflammation cytokines may contribute to increased TRAIL expression on NK cells [1, 8]. Then we checked the serum levels of main inflammation cytokines (TNFα, IL-1β, IL-10, IL-8, IL-6, and IL-12p70) in HC, CHB and HBV-ACLF patients by using CBA Inflammation kit. The results showed that serum levels of IL-6 and IL-8 were significantly elevated in patients with HBV-ACLF than those in HC and CHB patients (all $p < 0.001$, Fig.3A). However, the concentrations of other cytokines showed no significant differences among three groups (data not shown). We further determined the correlations between IL-6 or IL-8 levels and TRAIL expression. Significant correlations existed between frequency of TRAIL expression on NK cells and both serum IL-6 levels ($r = 0.346$, $p = 0.029$,

Fig.3B left) and IL-8 levels (r = 0.519, $p = 0.001$, Fig.3B right).

In order to further confirm the correlation between cytokines levels and TRAIL expression, PBMCs from 10 ACLF and 10 CHB patients were isolated and stimulated with IL-6 (10ng/ml) or IL-8 (10ng/ml) for 24 hours. As shown in Fig.3C left, the frequency of TRAIL expression on NK cells was increased from 12.5% to 20.2% after IL-6 stimulation, and 22.1% after IL-8 stimulation in ACLF patients. NK cells were activated after IL-6 or IL-8 stimulation (CD69 expression increased from 20.5% to 31.2% and 33%, respectively. Fig.3C right). Meanwhile, IL-6/IL8 incubation also increased TRAIL and CD69 expression on NK cells from CHB patients (Fig.3C). The results indicated that inflammation cytokines may contribute to NK cell activation and increased cytotoxicity.

3.5. NK cells from patients can induce TRAIL-mediated hepatocytes apoptosis

Next, we want to test whether NK cells from CHB and HBV-ACLF patients could cause hepatocytes apoptosis through TRAIL. First, purified NK cells from CHB and HBV-ACLF patients were incubated with or without 10ng/ml IL-6 and IL8 for 24 hours. Then apoptotic analysis of L02 cells with or without NK cells incubation were performed using annexin V/7-AAD double staining by Flow cytometry (Fig.4A). The results revealed that NK cells from HBV-ACLF patients caused a significant increase of apoptotic L02 cells in comparison with non-NK control (9.8% *vs* 2.1%, $p < 0.05$); when L02 cells incubated with cytokine-treated NK cells, apoptotic cells were furtherly increased to 19.9% ($p < 0.05$ *vs* untreated-NK cells, Fig 4A). NK cells from CHB patients didn't induce L02 cell going apoptosis (2.5% *vs* 1.9%, $p > 0.05$), however, the NK cells which treated with IL6/IL8 for 24 h could cause L02 cells apoptosis, the frequency of apoptotic cells was increased from 1.9% to 12.3% ($p <$

0.05). Second, purified NK cells from CHB and HBV-ACLF patients were incubated with IL-6 and IL8 for 24 h, simultaneously; L02 cells were cultured with 10ng/ml IL-6 and IL8 for 24 h. Then NK and L02 cells at an E/T ratio of 10:1 were incubated together for 6 h, with or without a TRAIL blocking antibody in the NK cells. The results showed that cytokine-treated NK cells increased the frequency of apoptotic cells in IL-6&IL8-stimulated L02 cells (45.5% *vs* 2.2% in ACLF patients; 30.1% *vs* 1.6% in CHB patients, $p < 0.001$. Fig 4B), indicating IL-6 and IL-8 stimulation sensitized human hepatocytes to high TRAIL-expressed NK cells induced apoptosis. Meanwhile the proportion of apoptotic cells was significantly reduced when L02 cells incubating with TRAIL blocking antibody-treated NK cells (16.3% *vs* 43.5% in ACLF patients; 15.1% *vs* 30.1%, $p < 0.001$. Fig 4B), suggesting NK cells caused hepatocytes apoptosis partially through TRAIL pathway. In addition, stimulation of IL-6 and IL-8 for L02 cells didn't change the frequency of apoptotic cells (2.1% *vs* 2.2%, Fig.4). Meanwhile, IL-6 and IL-8 incubation also increased the NK cells cytotoxicity from HCs (data not shown).

3.6. NK cells induced hepatocytes apoptosis via the caspase cascade pathway

We further measured whether the degree of cell apoptosis via the caspase cascade pathway used by TRAIL. Cytokine-treated NK cells from HBV-ACLF patients with or without TRAIL blocking antibody incubation were added to the cytokine-treated L02 cells, the degree of L02 cell caspase activation was assessed by flow cytometry. As shown in the Fig.4C, NK cells incubation induced fourfold increased caspase activations of L02 compared with non-NK control ($p < 0.05$). When TRAIL was blocked there was a reduction in NK cells-induced caspase activation compared with the non-antibody treated cells ($p < 0.05$). The results suggested that

TRAIL may play a major role in the NK cells-induced caspase activation.

4. Discussion

HBV-related acute-on-chronic liver failure is characterized as massive hepatic cell death and high mortality. HBV is a non-cytopathic virus which mediates liver injury by the host's immune responses. In chronic HBV infection, HBV-specific T cells (both CD4+ and CD8+) which expressed programmed death 1 (PD-1) molecules are exhausted and functionally impaired [8, 20-21]. NK cells represent 5-10% of peripheral blood lymphocytes and about 30% of lymphocytes in the healthy human liver. An increase of active NK cells in the liver has been observed in patients with immune-activated CHB [15] and HBV-ACLF [17]. The patients with HBV-ACLF in our study exhibited decreased total circulating NK frequencies but increased NK activation status. Furthermore, the percentage of the $CD3^{-}CD56^{bright}$ subset was increased in HBV-ACLF patients, indicating a re-distribution in peripheral NK cell component in patients with liver failure. The cause for the decreased peripheral NK cell frequency in HBV-ACLF patients is unknown. It's possible that circulating NK cells migrated to the diseased liver since the enrichment of NK cells was found in the liver of HBV-ACLF patients in previous reports [15, 17].

Previous reports have shown that liver NK cells may contribute to hepatocytes death mediated by TRAIL in CHB patients with hepatic flare [18]. Unfortunately, since liver biopsy is hard to get from liver failure patients, we couldn't compare the frequencies of TRAIL-expression NK cells in liver among HC, CHB and HBV-ACLF patients. The other finding in this study was that TRAIL expression mainly on $CD56^{bright}$ subset of circulating NK cells. It is believed that $CD56^{bright}$ NK cells are mainly cytokine-producing cells whereas $CD56^{dim}$ NK cells are considered to be more

cytotoxic [20]. The $CD56^{bright}$ subset of NK cells comprise the minority in the peripheral blood and are considered as the main cytokine producers [22, 23]. Since hepatocytes are considered to be relatively resistant to cytotoxicity of NK cells via the perforin/granzyme pathway, $CD56^{bright}$ subset is likely to play a major role in hepatocellular damage. Meanwhile, viral infections may alter these proportions, leading to a relative enrichment of the $CD56^{bright}$ population [16, 24]. In this study, we suggested that the increase of TRAIL-expression $CD56^{bright}$-NK may contribute to liver injury of patients with HBV-ACLF.

The role of TRAIL in hepatic cell death is controversial. Initial studies suggested that TRAIL, in contrast to TNF and FasL, induce apoptosis in various tumor cells but not normal cells in vivo [25, 26]. However, studies in mouse models of liver disease have reinforced the notion of NK-expressed TRAIL inducing damage of nonmalignant tissues in vivo, showing TRAIL-dependent death of hepatocytes [27, 28]. Furthermore, normal human hepatocytes are susceptible to TRAIL-induced apoptosis in vitro [25, 29]. In this study, activated NK cells from HBV-ACLF patients caused about 10%-20% of L02 cells going apoptosis, suggesting high TRAIL-expressed NK cells do have cytotoxicity to normal human hepatocytes. It has been suggested that susceptibility to TRAIL-induced liver damage may be increased under inflammatory conditions [18, 30-31). Cytokines are essential mediators, which regulate inflammatory responses. Expression profiles of cytokines such as IFN-α and IL-12/IL-15/IL-18 may also be involved in the regulation of NK cell activity in chronic HCV and HBV infection [32, 33]. During hepatic flare in ACLF, inflammatory cytokines are abundant in the patients' serum and liver as reported before [34-36]. We investigated that serum levels of IL-6 and IL-8 were significantly elevated and positively correlated with TRAIL-expression on NK cells in HBV-ACLF

patients. Moreover IL-6 and IL-8 stimulation in vitro could increase TRAIL expression and NK cells activation, indicating that inflammatory circumstance of ACLF may contribute to NK cell cytotoxicity to hepatocytes through up-regulating TRAIL expression. To further confirm the roles of cytokines for hepatocytes apoptosis, the L02 cells pretreated with IL-6 and IL-8 for 24 hours before incubation with NK cells from HBV-ACLF patients. The results showed that apoptotic L02 cells almost doubled after cytokine treatment, supporting our hypothesis that inflammatory circumstance in HBV-ACLF patients sensitized hepatocytes apoptosis. Furthermore, L02 cell apoptosis was almost half abolished by the TRAIL-neutralizing antibodies, indicating that it was partially mediated by TRAIL. Caspase activation is a common feature of cell death through apoptosis. NK cells strongly induced cell apoptosis and caspase activity in IL-6&IL-8 stimulated hepatocytes in this study. The detail mechanism need to be further investigated.

In conclusion, our data showed that peripheral NK cells from HBV-ACLF patients expressed higher levels of TRAIL. Inflammation circumstance of ACLF increased TRAIL expression and activation levels of peripheral NK cell. TRAIL-expressed NK mediated hepatocyte cytotoxicity and IL-6/IL-8 stimulation sensitized human hepatocytes to TRAIL induced apoptosis. The limitation of our study is that we used L02 cells but not primary hepatocytes for cytotoxicity analysis. L02, a human immortal hepatocyte cell line, isn’t a transformed cell. It maintains the most morphologic and biological characteristics of primary hepatocyte and was used in the previous studies. The L02 cells provide a convenient model to discuss the mechanisms of activation of TRAIL pathway. Our data in this manuscript might only suggest that activated NK cells of ACLF can kill normal hepatocytes. The mechanism should be confirmed in primary hepatocytes in the further studies.

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Figure legend

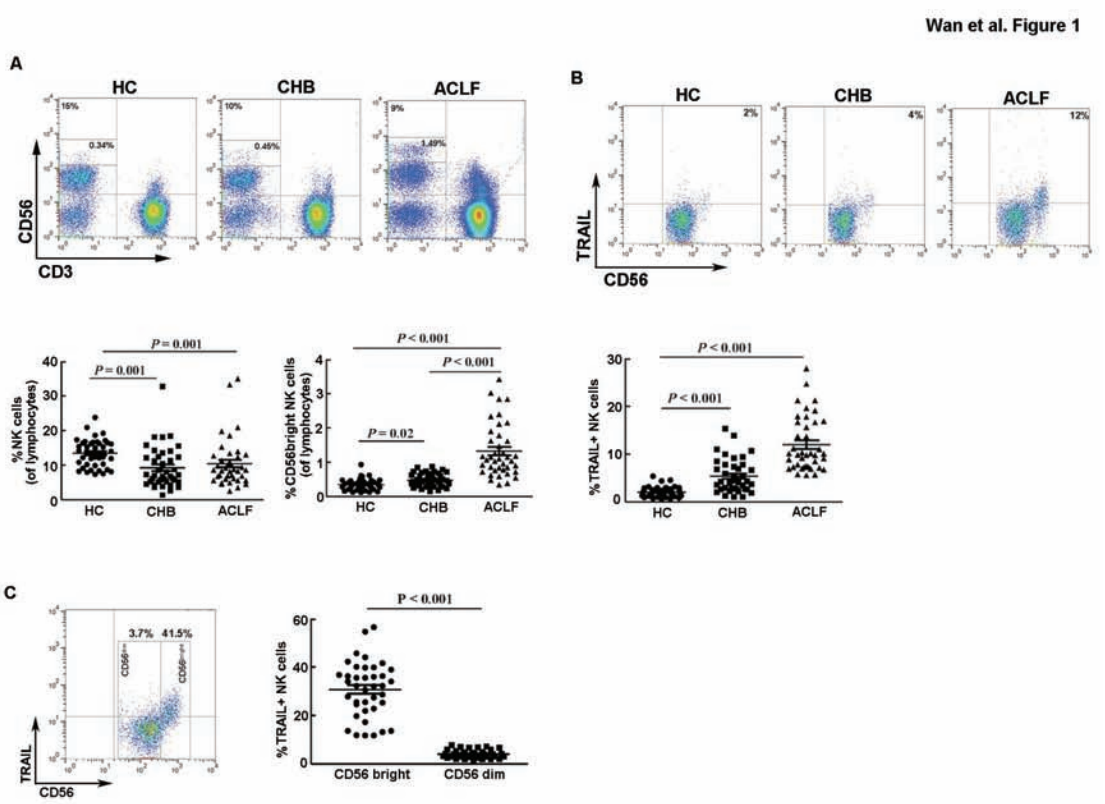
Fig. 1 Elevated TRAIL expression on $CD3^-CD56^{bright}$ NK cells in patients with HBV-related acute-on-chronic liver failure. (A) Representative flow cytometry analysis of CD3 and CD56 expression on lymphocytes. Lymphocytes were first gated according to forward/side scatter analysis. Then NK cells were identified by $CD3^-$ and $CD56^+$ staining. The numbers in quadrant indicated the frequency of CD3-CD56+ and $CD3\text{-}CD56^{bright}$ NK cells of total lymphocytes, respectively. Blow is a comparison of the frequencies of $CD3^-CD56^+$ and $CD3^-CD56^{bright}$ NK cells among HC (n = 40), CHB (n = 40) and HBV-ACLF patients (n = 40). (B) Representative flow cytometry analysis of TRAIL expression on $CD3^-CD56^+$ NK cells. Pooled data showed a comparison of the frequency of $TRAIL^+$ NK cells among different groups. (C) Representative flow cytometry analysis comparing TRAIL expression on $CD3^-CD56^{bright}$ and $CD3^-CD56^{dim}$ NK cell subsets. The percentages of TRAIL+ NK cells in each cell subset are presented on the right.

Fig. 2 NK cells from patients with HBV-ACLF displayed increased activation status. (A) Representative flow cytomere analysis of CD69 expression on NK cells among HC (n = 40), CHB (n = 40) and ACLF patients (n = 40). (B) Summary data indicated the percentage of $CD69^+$ NK cells among three groups. The data represent the mean ± SD. *P* values are shown.

Fig. 3 Elevation of inflammation cytokines in the serum of patients with HBV-ACLF and positively correlated with TRAIL expression on NK cells. (A) Serum cytokine levels were analyzed in 40 healthy controls, 40 CHB and 40 HBV-ACLF patients by CBA Inflammation kit. Pooled data indicated the levels of IL-6 (left) and IL-8 (right) among three groups. (B) Correlations between levels of IL-6 (left) or IL-8 (right) and percentage of TRAIL+ NK cells in patients with

HBV-ACLF. (C) PBMCs from ACLF and CHB patients were ex vivo stimulated with IL-6 (10ng/ml) or IL-8 (10ng/ml) for 24 h, frequency of TRAIL+ NK cells (left) and CD69+ NK cell (right) were determined by flow cytometry analysis (*, #: $P < 0.05$ *vs.* no cytokine control, respectively).

Fig.4 IL-6 and IL-8 stimulation sensitized human hepatocytes to NK cell induced apoptosis. (A) Purified NK cells from 10 HBV-ACLF and 10 CHB patients were incubated with or without 10ng/ml IL-6 and IL8 for 24 h. NK cells and L02 cells at an E/T ratio of 10:1 were incubated together for 6 h. L02 cells were collected and subjected to annexin V/7-AAD double staining, followed by flow cytometry analysis. Numbers in each quadrant indicate the percentage of cells that were annexin V^+/7-AAD^- (early apoptosis cells) and annexin V^+/7-AAD^+ (late apoptosis cells). The percentage of apoptotic cells were summarized on the right (*: $P < 0.05$, **: $P < 0.01$ *vs* non-NK control in ACLF; $^{\#}$: $P < 0.05$ *vs* non-NK control in CHB). (B) NK cells from HBV-ACLF and 10 CHB patients were incubated with 10ng/ml IL-6 and IL8 for 24 h, simultaneously; L02 cells were cultured with 10ng/ml IL-6 and IL8 for 24 h. Then NK and L02 cells at an E/T ratio of 10:1 were incubated together for 6 h, with or without a TRAIL blocking antibody in the NK cells. Summary data of percentage of apoptotic cells were shown on the right (*, $^{\#}$: $p < 0.05$ *vs* non-NK control; **, $^{\#\#}$: $p < 0.05$ *vs* NK (+)-treated group). (C) L02 cells and NK cells from HBV-ACLF patients were incubated with 10ng/ml IL-6 and 10ng/ml IL-8 for 24 h, respectively. NK cells with or without TRAIL blocking antibody pre-incubation were added to L02 cells at an E/T ratio of 10:1 for 6 h before visualization of caspase activation with the carboxyfluorescein-labeled FAM-VAD-FMK and detected by flow cytometry (expressed as mean fluorescence intensity). (*: $p < 0.05$ *vs* NK (-), $^{\#}$: $p < 0.05$ *vs* NK (+)-treated)

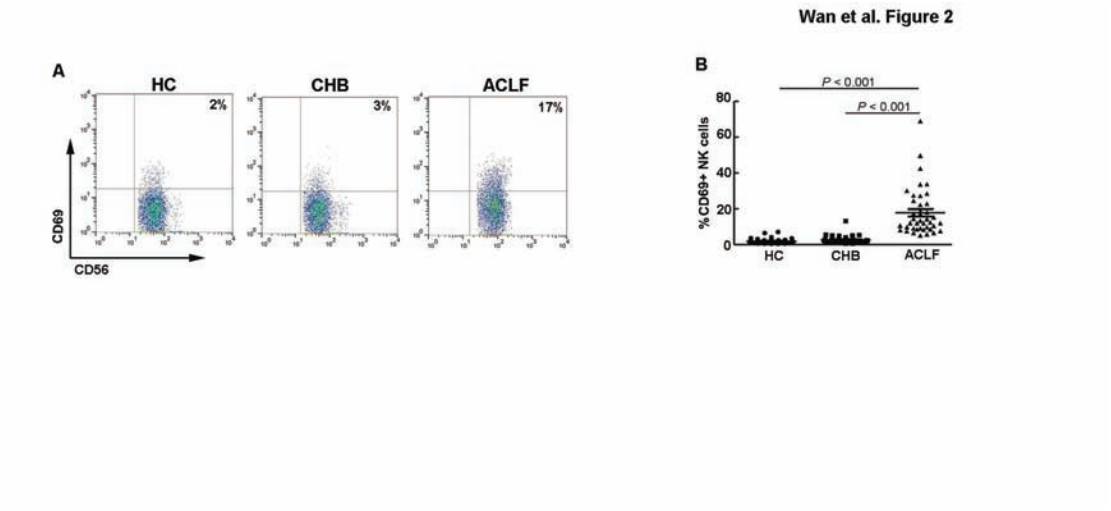

Wan et al. Figure 1
A
HC
CHB
ACLF
15%
0.34%
10%
0.46%
9%
1.49%
CD56
CD3
B
HC
CHB
ACLF
2%
4%
12%
TRAIL
CD56
% NK cells (of lymphocytes)
P = 0.001
P = 0.001
HC
CHB
ACLF
% CD56bright NK cells (of lymphocytes)
P < 0.001
P < 0.001
P = 0.02
HC
CHB
ACLF
% TRAIL+ NK cells
P < 0.001
P < 0.001
HC
CHB
ACLF
C
3.7%
41.5%
CD56bright
CD56dim
TRAIL
CD56
% TRAIL+ NK cells
P < 0.001
CD56 bright
CD56 dim

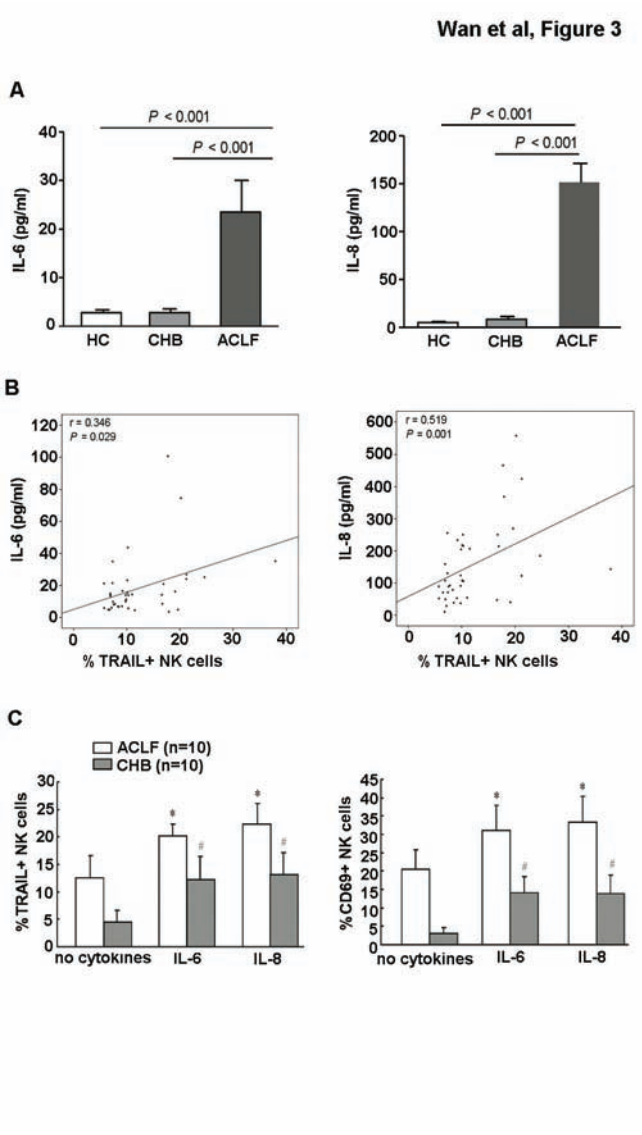
1

2

3

4


Wan et al. Figure 2
A
HC
CHB
ACLF
2%
3%
17%
CD69
CD56
B
P < 0.001
P < 0.001
% CD69+ NK cells
80
60
40
20
0
HC
CHB
ACLF


Wan et al, Figure 3
A
P < 0.001
P < 0.001
IL-6 (pg/ml)
HC
CHB
ACLF
P < 0.001
P < 0.001
IL-8 (pg/ml)
HC
CHB
ACLF
B
r = 0.346
P = 0.029
IL-6 (pg/ml)
% TRAIL+ NK cells
r = 0.519
P = 0.001
IL-8 (pg/ml)
% TRAIL+ NK cells
C
ACLF (n=10)
CHB (n=10)
%TRAIL+ NK cells
no cytokines
IL-6
IL-8
%CD69+ NK cells
no cytokines
IL-6
IL-8