Clinical and Experimental Medicine (2019) 19:93–104
https://doi.org/10.1007/s10238-018-0534-1

ORIGINAL ARTICLE

Myeloid cell-like transcript 2 is related to liver inflammation and the pathogenesis of hepatitis B via the involvement of CD8$^+$T cell activation

Jun-Chi Xu[1,2,3] · Fei Gao[4,5] · Yi-An Liu[1,2] · Xiao-Long Zhang[6] · Hui Chen[1,2,3] · Xiao-Yan Zhu[1,2,3] · Hua-Feng Song[1,2,3] · Feng Qian[1,3] · Ming Li[1,3] · Chen Yang[4,5] · Chuan-Wu Zhu[1,2,3] · Ping Xu[1,2,3]

Received: 2 July 2018 / Accepted: 18 October 2018 / Published online: 25 October 2018

Abstract

This study analysed the biological significance of TLT-2 on CD8$^+$T cells in hepatitis B patients and provided a theoretical basis for the potential role of TLT-2 as an immune regulator. Flow cytometry sorting, isobaric tags for relative and absolute quantitation and short hairpin RNAs were used to analyse the function of TLT-2 on CD8$^+$T cells in hepatitis B patients. The TLT-2 expression levels in the acute hepatitis B and chronic hepatitis B groups were significantly higher than that in the healthy control group and were positively correlated with ALT and AST. The CD8$^+$TLT-2$^+$T cells exhibited stronger immune function and greater cell proliferation ability and secreted higher levels of cytokines than the CD8$^+$TLT-2$^-$T cells. An analysis of the proteome differences between the TLT-2$^+$CD8$^+$T and TLT-2$^-$CD8$^+$T cells revealed that TLT-2 affected CD8$^+$T cell activation by regulating Granzyme B expression and by further action on the NF-κB signalling pathway. This study first elucidated the mechanism by which TLT-2 influences the activation of CD8$^+$T cells, improved the understanding of the TLT-2 signalling pathway and clarified the role of the TLT-2$^+$CD8$^+$T cell subset in hepatitis B virus infection. The study proposed a novel subset of CD8$^+$T cells that could be useful for understanding the immune function of patients with hepatitis B and further elucidating the pathogenesis of hepatitis B by analysing changes in this subpopulation with the goal of providing a new target for the treatment of hepatitis B.

Keywords Inflammation · TLT-2 · CD8$^+$T · Cell activation · Costimulation

Abbreviations

HBV	Hepatitis B virus
TREM	Triggering receptors expressed by myeloid cells
TLT-2	Trem (triggering receptor expressed on myeloid cells)-like transcript 2
HC	Healthy controls

Jun-Chi Xu, Fei Gao and Yi-An Liu have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10238-018-0534-1) contains supplementary material, which is available to authorized users.

✉ Chen Yang
yangchen_coffee@126.com

✉ Chuan-Wu Zhu
zhuchw@126.com

✉ Ping Xu
xuping19670822@126.com

[1] Department of Hepatology, The Fifth People's Hospital of Suzhou, 10, Guangqian Road, Suzhou 215000, Jiangsu Province, People's Republic of China

[2] Key Laboratory of Infection and Immunity of Suzhou City, Suzhou, People's Republic of China

[3] The Affiliated Infectious Disease Hospital of Soochow University, Suzhou, People's Republic of China

[4] The Affiliated Suzhou Hospital of Nanjing Medical University, 26 Daoqian Road, Suzhou 215000, Jiangsu Province, People's Republic of China

[5] Suzhou Municipal Hospital, Suzhou, People's Republic of China

[6] Suzhou Center for Disease Prevention and Control, Suzhou, People's Republic of China

AHB	Acute hepatitis B
CHB	Chronic hepatitis B
HBV-LC	Hepatitis B virus-associated liver cirrhosis
HBV-HCC	Hepatitis B virus-associated hepatocellular carcinoma
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
TBIL	Total bilirubin
ALB	Albumi
α-TNF	Tumor necrosis factor α
CT	Computed tomography
MRI	Magnetic resonance imaging
HBeAg	Hepatitis B e-antigen
PBMCs	Peripheral blood mononuclear cells
SSC	Side scatter
FSC	Forward scatter
iTRAQ	Isobaric tags for relative and absolute quantitation
LC–MS/MS	Liquid chromatography coupled to tandem mass spectrometry
GO	Gene ontology
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
AP-1	Activator protein 1
PMA	Phorbol-12-myristate-13-acetate
BID	BH3 interacting-domain death agonist
ns	Not significant
shRNA	Short hairpin RNA

Introduction

Hepatitis B virus (HBV) is a type of hepadnavirus. There are approximately 240 million people with chronic hepatitis B or carrying HBV worldwide. Chronic infection with HBV is a main factor that leads to cirrhosis and liver cancer. Every year, approximately 3 million chronic HBV-infected persons die of chronic hepatitis B, cirrhosis and liver cancer [1, 2]. However, the pathogenesis of chronic hepatitis B remains unclear. Abnormal numbers and dysfunctions of T cells in patients with HBV infection have been found to be closely related to the occurrence, development and chronicity of hepatitis B [3–5]. Therefore, it is necessary to discover additional immune molecules that lead to liver inflammation and are related to the pathogenesis and clinical outcome of HBV patients.

The triggering receptor expressed by myeloid cells (TREM) is a gene family that was first identified by Bouchon in 2000 [3]. TREM mainly includes the following four family members: TREM-1 [3, 4], TREM-2 [5, 6], TLT-1 [7, 8] and TLT-2 [3]. The TREM family is mainly expressed on the surface of myeloid-derived cells; thus, the important roles of TREMs and TREM receptors in the regulation of inflammatory responses warrant further study [9].

TLT-2 is a novel member of the TREM family. TLT-2 is mainly expressed in B cells and is also obviously expressed in newly isolated $CD8^+$T cells that have not been stimulated, as well as in $CD4^+$T and $CD8^+$T cells upon short-term stimuli [10–12]. Moreover, large groups of cells express TLT-2 in the spleen, lymph nodes, blood, bone marrow and abdominal cavity. TLT-2 is expressed in the early stage of B cell development and can be detected in all developing B cells and peripheral B cells in the mouse [11]. Additionally, TLT-2 is expressed in mouse neutrophils and monocytes [13]. In response to in vivo inflammation, the TLT-2 gene is up-regulated in neutrophils and macrophages but is not changed on the surfaces of B cells. The special expression mode of TLT-2 indicates its involvement in the immune response and a regulatory role in the inflammatory response. In 2008, Hashiguchi et al. [14] found that TLT-2 exhibits a high degree of similarity with members of the CD28 family. Because CD28 is the receptor of B7, these authors studied whether B7-H3 combines with TLT-2 and found that B7-H3 is the ligand of TLT-2 and can promote the activation of T cells [12, 14]. However, Leitner [15] found that there is no specific binding between the two molecules based on flow cytometry and suggested that B7-H3 exerts a negative effect on T cell activation [16–19]. Subsequently, Vigdorovich used bioinformatics to prove that there are no binding sites between B7-H3 and TLT-2 [20]. Therefore, the ligand of TLT-2 remains to be researched and determined.

In recent years, TLT-2 has gradually become the hotspot of the TREM family. The mechanism of function and relationship of TLT-2 with the disease are constantly being elucidated. Recent researches [21–25] found that TLT-2 is stored in human neutrophil primary granules and is up-regulated in response to inflammatory mediators, and Halpert proved that TLT-2 potentiates neutrophil antibacterial activity and chemotaxis in response to G protein-coupled receptor-mediated signalling. In another study [13], TLT-2 was proven to be closely related to Alzheimer's disease. Unlike its expression on the neutrophil surface, TLT-2 expression increases with $CD8^+$T cell activation [10–12, 14]. However, the correlations of TLT-2 expression on $CD8^+$T cells with human diseases have not yet been studied. Therefore, we systematically analysed the relationship between TLT-2 and HBV infection and demonstrated the action of TLT-2 on CTL in human diseases. Moreover, our findings are helpful for revealing the role of this molecule in the immune response, which is beneficial for understanding the immune statuses of HBV patients. The results further illuminate the immune pathogenesis of hepatitis B and may potentially be used to control the severity of liver inflammation in the treatment of HBV patients.

Materials and methods

Study participants

The recruited samples included healthy donors ($n = 20$) and patients with acute hepatitis B (AHB, $n = 25$), chronic hepatitis B (CHB, $n = 83$), hepatitis B virus-associated liver cirrhosis (HBV-LC, $n = 22$) and hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC, $n = 30$). None of the patients were coinfected with hepatitis C virus (HCV), hepatitis D virus (HDV) or human immunodeficiency virus (HIV), and patients with any other liver diseases were also excluded. The healthy donors were tested and found to be negative for the markers of HBV, HCV, HDV and HIV infection. AHB was diagnosed according to ALT elevation, hepatitis B e-antigen (HBeAg) seroconversion, hepatitis B surface antigen (HBsAg) seroconversion, the appearance of anti-HBc IgM, the disappearance of HBV-DNA and the lack of any liver disease history. CHB was defined by persistent HBsAg positivity for at least 6 months and elevated ALT levels. The diagnosis of HBV-LC was mainly dependent on liver ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) and some serological fibrosis tests. HBV-HCC was diagnosed according to an elevated a-fetoprotein level of ≥ 500 ng/ml, liver ultrasound, CT imaging or MRI [26]. This study was approved by the Institutional Review Board of the Fifth People's Hospital of Suzhou, and informed consent was obtained from all participants and healthy donors before their enrolment in this study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All methods were performed in accordance with relevant guidelines and regulations.

Detection of HBV serological and virological markers and liver function tests

HBV serological markers, including HBsAg, HBeAg, antibodies to HBsAg and HBeAg, anti-HBc IgM and IgG, were detected using a Chemiluminescent Microparticle Immunoassay (Abbott Ireland, Diagnostics Division, Sligo, Ireland). HBV-DNA was quantified with a Roche LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland) and its reagents (Sansure Biotech, Ltd., Hunan, China). A standard automatic biochemistry analyser (Hitachi, Tokyo, Japan) and reagents (Wako Pure Chemical Industries, Ltd., Japan) were employed for the liver function tests, which included tests for ALT, AST, ALB and TBIL.

Flow cytometric analysis

Monoclonal antibodies for CD3-Pacific Blue (clone UCHT1), CD8-Alexa Fluor 700 (clone RPA-T8), Granzyme B-FITC (clone B18.1.), Tim-3-APC-eFluor780 (clone F38-2E2), CD69-PE-Cyanine7 (clone FN50) and PD-1-PerCP-eFluor710 (clone eBioJ105) were purchased from eBioscience (San Diego, CA, USA). TLT-2-PE was purchased from Biolegend (clone MIH61, San Diego, CA, USA). For each test, 100 µL of fresh heparinized whole blood from the patients or healthy donors was lysed with FACSTM lysing solution (BD Biosciences, San Jose, CA, USA), washed with phosphate-buffered saline, stimulated with PMA (10 ng/ml) and ionomycin (1 µg/ml) for 4 h and incubated for the last 3 h with brefeldin A (10 µg/ml). The cells were transferred to a U-bottom plate, stained with surface marker antibodies in HBSS containing 1% FCS, fixed with 2% formaldehyde and permeabilized with 0.5% saponin. The cells were stained with anti-Granzyme B, washed with phosphate-buffered saline, fixed and eventually detected with a BD FACSAria with BD FACSDiva (San Jose, CA, USA) software support. The data were analysed using the FlowJo software (Tree Star Inc., Ashland, OR, USA).

$CD8^+TLT\text{-}2^+$ and $CD8^+TLT\text{-}2^-$T cell proliferation assays

The peripheral blood mononuclear cells (PBMCs) of acute hepatitis B patients were freshly isolated from heparinized venous blood using Ficoll and stimulated with PMA and ionomycin for 24 h. Then, the cells were stained with CD3-Pacific Blue, CD8-Alexa Fluor 700 and TLT-2-PE mAbs, and the $CD8^+TLT\text{-}2^+$ and $CD8^+TLT\text{-}2^-$T cell subsets were sorted by flow cytometry (BD Aria II Cell Sorting System). The purified $CD8^+TLT\text{-}2^+$ (2×10^5/mL) and $CD8^+TLT\text{-}2^-$ (2×10^5/mL) cells were divided into subgroups according to the incubation time (12 h, 24 h or 48 h) and seeded into 96-well plates (100 µl/well). Each subgroup set-up included six wells, and the plates were placed in an incubator with 5% CO_2 at 37 °C. After 12 h, 24 h and 48 h, the reagent of the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added to the culture system and incubated for 5 h. The absorbance of each well was measured at 450 nm with a microplate reader (Bio-Rad). Simultaneously, the supernatants of the cell cultures were collected from each well for ELISA assays.

shRNA knocks down the TLT-2 expression on the $CD8^+$T cells

Peripheral blood mononuclear cells were isolated with Ficoll from 400 mL of healthy human peripheral blood. $CD8^+$T cells were sorted with a MicroBead kit and counted. Next,

1000 U/ml of IL-2 was added for 24 h. The shRNA virus particles targeting TLT-2 were added and mixed, and then, the samples were placed into a 5% CO_2 incubator at 37 °C, and 1000 U/ml IL-2 was added every 3 days. The cells were cultured for 5 days. GFP-positive cells were sorted by flow cytometry followed by the addition of PMA and ionomycin stimulation. The expression of TLT-2 was detected by Western blot and qPCR. Cell proliferation was detected with a CCK8 kit, and the secretions of the cytokines IFN-γ and TNF-α were detected by ELISA at 12 h, 24 h and 48 h. In this experiment, $CD8^+$T cells that were transfected with empty vector were used as the control group. The siRNA sequence for TLT-2 was as follows: TLT-2 siRNA: 5′-UUU UUGUAGCCCUUAUAGGAG-3′.

Enzyme-linked immunosorbent assay (ELISA)

The cell cultures were centrifuged at 3000 rpm for 15 min, and the cell-free supernatant from each well was obtained and stored at − 20 °C. The levels of TNF-β and IFN-γ in the supernatant were analysed with ELISA kits (BlueGene, Shanghai, China) according to the manufacturer's instructions.

Protein electrophoresis and Western blot analysis

To resolve the proteasome subpopulations in the cell extracts, 5% gradient gels were prepared as previously described. SDS–PAGE was performed using 12% gel, and the resolved proteins were then transferred to a PVDF membrane. The antibodies against NF-κB (p100/p52), NFATc1, Granzyme B (GZMB) and BID were purchased from Biolegend (San Diego, CA, USA), and the antibody against AP-1 was purchased from R&D (Minneapolis, MN, USA). Anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase (HRP) secondary antibodies (Sigma-Aldrich) were used, and ECL detection was subsequently performed (Thermo Scientific, Waltham, MA, USA).

Quantitative RT-PCR

The total RNA was isolated from the transfected cells using RNeasy Mini Kits (Qiagen, Tokyo, Japan) and RNAiso (Takara Bio, Shiga, Japan). The RNA quality was assessed with BioDrop uLITE PC (Bio-Rad, USA). cDNA was synthesized using a PrimeScript RT Master Mix Kit (Takara Japan). The quantitative RT-PCR analyses were performed on a c1000 Touch Thermal Cycler (Bio-rad, USA) System using SYBR Premix Ex Taq (Perfect Real Time, Takara). The primer sequences for GAPDH were 5′-ATCATCCCT GCCTCTACTGG-3′ and 5′-TTTCTAGACGGCAG GTC AGGT-3′, and those for TLT-2 were 5′-CCTCTCGAG ATGGCCCCAGCCTT CCTGC-3′ and 5′-TCGGATCCA GTAGACTTCCACATAGG-3′. All experiments were performed in triplicate and repeated in at least three separate experiments.

iTRAQ and LC–MS/MS analyses

iTRAQ labelling and SCX chromatography labelling of the samples with the iTRAQ and strong cation exchange chromatography (SCX) methods were performed as previously described. Briefly, the PBMCs were isolated with Ficoll from 40 mL of healthy human peripheral blood and stimulated with PMA and ionomycin for 24 h. the $CD8^+$TLT-2^+T cells and $CD8^+$TLT-2^-T cells were separated and collected by flow cytometry, and the protein lysate (8 M urea, 1% SDS) was mixed with the cocktail and vortexed, added to the cells at a volume appropriate for the ratio of 1:8, split for 30 min by placement on an ice shaker and sonicated for 2 min. After centrifugation at 4.4 °C and 16,000×*g*, the supernatant was obtained. The protein concentration was quantified by the Bradford or BCA method. The protein samples were analysed by SDS–PAGE to evaluate whether the sample qualities met the requirements of the follow-up experiments. The protein samples that met the standard were reductively alkylated. Equal amounts of protein taken from each sample were enzymatically hydrolysed with trypsin. The peptides were labelled with iTRAQ reagent. The labelled peptides were mixed in equal amounts. The mixed peptides were pre-separated using a C18 reverse-phase column. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) analysis was performed, and the Sequest module of the Proteome Discoverer or Mascot was used for comparisons with the database (www.uniprot.org/proteomes). The search results were used for the statistical and bioinformatics analyses.

Bioinformatics analysis

A brief description of the basic information analysis procedure for quantitative proteomics is as follows: the peak identification was performed on the original file obtained by mass spectrometry to obtain a peak list, and a reference database was established for the identification of the peptides and proteins. All identified proteins were annotated with the Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG http://www.genome.jp/kegg/) pathways. The differential proteins were screened according to the difference multiples and significant *p* values. (The screening criteria for the significantly differentially expressed proteins in this project were defined as FC < 0.67 or FC > 1.50.) The differential proteins were analysed for presentation as a volcano map and a heatmap of the differential proteins. The differentially expressed proteins were annotated with

GO and KEGG, and Goatools (https://github.com/tanghaibao/GOatools) was used for the profiling enrichment analysis. Fisher's method was used for significance testing. The STRING database (http://string-db.org/db.org/db.org/db.org/) was used for the analyses of the differential protein interaction networks.

Statistical analysis

The data were analysed with GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) and summarized and are presented as the means ± the standard deviations (SDs). For the comparisons of the collected data, a nonparametric test (Mann–Whitney *U* test), Student's t test and the Chi-square test were performed, and a two-tailed $p < 0.05$ was considered statistically significant. A nonparametric correlation test (Spearman) was applied to analyse the correlations of the TLT-2 expression with the ALT and AST levels.

Results

Clinical baseline characteristics of study populations

The recruited participants in this study were classified into the following five groups: HC, AHB, CHB, HBV-LC and HBV-HCC. Table 1 presents the ages, genders, HBeAg serum statuses, serum HBV-DNA concentrations and ALT, AST, ALB and TBIL levels. Based on the Child–Pugh classification [26], the 22 LC patients comprised 10 cases in class A, 5 cases in class B, and 7 cases in class C. According to the Barcelona Clinic Liver Cancer Staging classification [27], 10 patients were stage A, 8 patients were stage B, and 12 patients were stage C in the HCC group.

TLT-2 expression on peripheral $CD8^+$T cells of the HBV-infected patients

To elucidate the TLT-2 expression patterns on the $CD8^+$T cells according to disease progression, AHB, CHB, HBV-LC and HBV-HCC patients, who represented different stages of HBV infection, were enrolled, and TLT-2 expression was investigated by flow cytometric analysis. The gating strategies for the $CD8^+$T cells are illustrated in Fig. 1a. Compared with the HC group (17.74 ± 1.549%), the percentage of circulating $CD8^+TLT\text{-}2^+$T cells was significantly increased in the AHB (33.38 ± 2.616%), CHB (30.54 ± 1.515%) and HBV-LC (23.87 ± 2.643%) groups, but not in the HCC group (21.94 ± 4.046%). Among the four groups of HBV-infected patients, pairwise comparisons revealed that the differences were significant between the AHB group and the other three groups, but were not significant for the CHB versus HBV-LC, CHB versus HBV-HCC or HBV-LC versus HBV-HCC comparisons (Fig. 1b).

The correlations of the TLT-2 expression levels on the $CD8^+$T cells from the AHB, CHB, HBV-LC and HBV-HCC subjects with the ALT and AST levels were further analysed. The results revealed that the TLT-2 expression levels on the $CD8^+$T cells of the AHB and CHB patients were positively correlated with the ALT and AST levels (Fig. 1c–d), and TLT-2 expression was negatively correlated with AST in the HBV-HCC patients (Fig. 1f). We also analysed the correlation between TLT-2 and hepatitis B virus load and e antigen, but no correlations were found (sFig. 1).

Table 1 Clinical characteristics of the study groups

Characteristic	HC group	AHB group	CHB group	HBV-LC group	HBV-HCC group
Cases	20	25	83	22	30
Child–Pugh, A/B/C	NA	NA	NA	10/5/7	18/11/1
BCLC, A/B/C	NA	NA	NA	NA	10/8/12
Age, yr	37.55 ± 2.843	40.16 ± 2.970	37.14 ± 1.183	54.82 ± 2.574	59.15 ± 2.514
Male/female	13/7	17/8	62/21	16/6	24/6
HBeAg, ±	NA	12/14	49/34	7/15	4/17
HBV-DNA, log10 copies/mL	NA	2.736 ± 0.2320	4.041 ± 0.2383	3.346 ± 0.4321	3.016 ± 0.3471
ALT, log10 IU/L	NA	2.471 ± 0.09991	1.680 ± 0.03824	1.718 ± 0.07518	1.876 ± 0.09809
AST, log10 IU/L	NA	2.695 ± 0.0953	1.790 ± 0.0534	1.637 ± 0.0733	1.769 ± 0.1033
ALB, g/L	NA	36.19 ± 0.852	41.56 ± 0.671	32.95 ± 1.145	34.61 ± 2.167
TBIL, mmol/L	NA	137.5 ± 10.56	21.50 ± 1.53	31.98 ± 5.60	82.70 ± 27.75

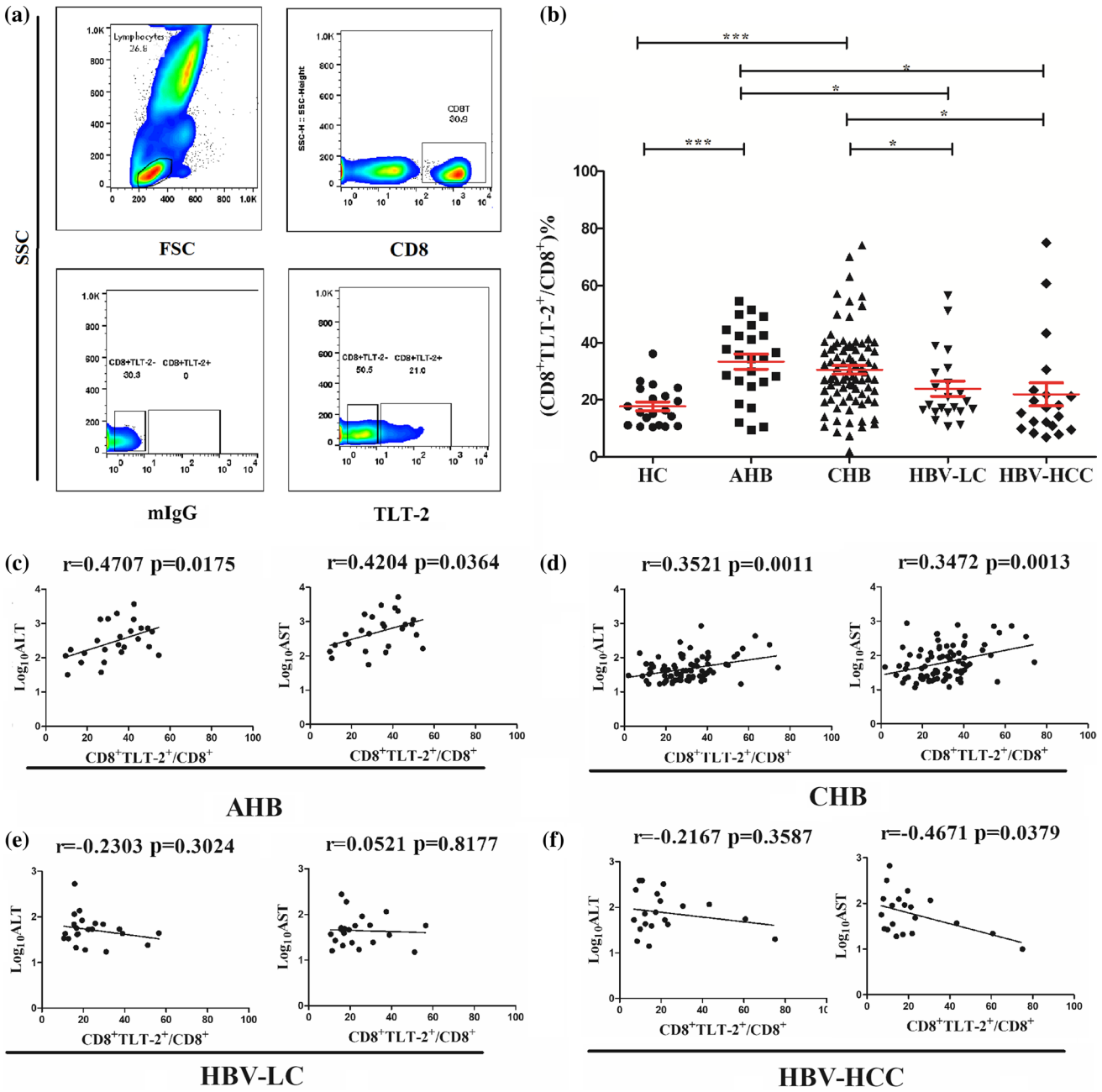

Fig. 1 Gating strategy and expression profiles for TLT-2 in the peripheral CD8$^+$T cells of the AHB, CHB, HBV-LC and HBV-HCC patients. **a** The gating strategies and representative results for TLT-2 expression on CD8$^+$T cells. **b** Statistical analyses of the TLT-2 expression in circulating CD8$^+$T cells from the HC, AHB, CHB, HBV-LC and HBV-HCC groups. **c–f** Correlation analyses of the relations of TLT-2 expression on the CD8$^+$T cells of patients with HBV infection with ALT and AST levels in the AHB, CHB, HBV-LC and HBV-HCC groups. *$p<0.05$; **$p<0.01$; ***$p<0.001$

Analysis of correlations between TLT-2 expression on CD8$^+$T cells and sB7-H3 in serum or B7-H3 expression on CD14$^+$ monocyte cells

The relationship of TLT-2 and B7-H3 is still in dispute and the B7-H3 is very important for T cell activation, so we analyse the relationship in hepatitis B and hope to understand the relationship, which will help to understand the role of T cell activation in host anti-HBV infection. The trend between TLT-2 expression on CD8$^+$T cells from AHB, CHB, HBV-LC and HBV-HCC subjects and sB7-H3 in serum or B7-H3 expression on CD14 monocytes cells is compared. The results showed that the B7-H3 and TLT-2 had similar changes in both AHB group and CHB group.

But in HBV-LC and HBV-HCC groups, the content of sB7-H3 and $CD14^+B7H3^+$ cell subgroup was further increased, while $CD8^+TLT\text{-}2^+T$ cells fell (sFig. 2). After analysing the TLT-2 and B7-H3 expression in HBV-infected patients, we found that the expressions of TLT-2 and B7-H3 were not synchronized. So, based on our results, we believe that B7-H3 is not the ligand of TLT-2.

$CD8^+TLT\text{-}2^+T$ cells exhibited stronger immune function

The changes in the phenotypes of the $CD8^+TLT\text{-}2^+T$ and $CD8^+TLT\text{-}2^-T$ cells were analysed by flow cytometry. The results revealed that, compared with the $CD8^+TLT\text{-}2^-T$ cell subset, CD69, i.e. markers of proliferation and activation, was highly up-regulated, and the inhibitory receptor PD-1 and Tim-3 were down-regulated, in the $CD8^+TLT\text{-}2^+T$ cell subset. Moreover, the $CD8^+TLT\text{-}2^+T$ cells also exhibited high levels of expression of the GZMB marker, which is considered to be a phenotypic marker of CTL (Fig. 2a). Figure 2b illustrates that the $CD8^+TLT\text{-}2^+T$ cells exhibited a faster proliferation rate (12 h: $p<0.001$, 24 h: $p<0.05$ and 48 h: $p<0.05$) and greater secretions of IFN-γ (12 h: $p>0.05$, 24 h: $p<0.05$ and 48 h: $p<0.05$, respectively) and TNF-α (12 h: $p<0.01$, 24 h: $p<0.01$ and 48 h: $p<0.05$, respectively) than the $CD8^+TLT\text{-}2^-T$ cells. Moreover, the cell proliferation and cytokine secretion increased with the time of cell culture.

Proteins that were differentially expressed between the $CD8^+TLT\text{-}2^+T$ and $CD8^+TLT\text{-}2^-T$ cells were analysed with proteomics

Regarding the further analysis of the function of TLT-2 in $CD8^+T$ cells, it was very straightforward and necessary to analyse the differences between the protein groups of the $CD8^+TLT\text{-}2^+T$ and $CD8^+TLT\text{-}2^-T$ cells. In this study, iTRAQ was used to analyse the purify $CD8^+TLT\text{-}2^+T$ and purify $CD8^+TLT\text{-}2^-T$ cells, and the proteomic differences between the two cell groups were screened using the $TLT\text{-}2^+CD8^+T$ cells as a standard. The screening criteria for significantly differentially expressed proteins were defined as FC ($CD8^+TLT\text{-}2^-T/CD8^+TLT\text{-}2^-T$) < 0.67 or > 1.50, and 399 proteins were identified as differentially expressed (sTable 1). There were 160 up-regulated proteins and 239 down-regulated proteins in the TLT-2 cells (Fig. 3a). According to the pattern revealed by clustering analysis, a heatmap was used to identify the significant differences in protein expression between the two groups (Fig. 3b). GO analysis was used to reveal that more than 20% of the differentially expressed proteins were involved in immune system processes (Fig. 3c).

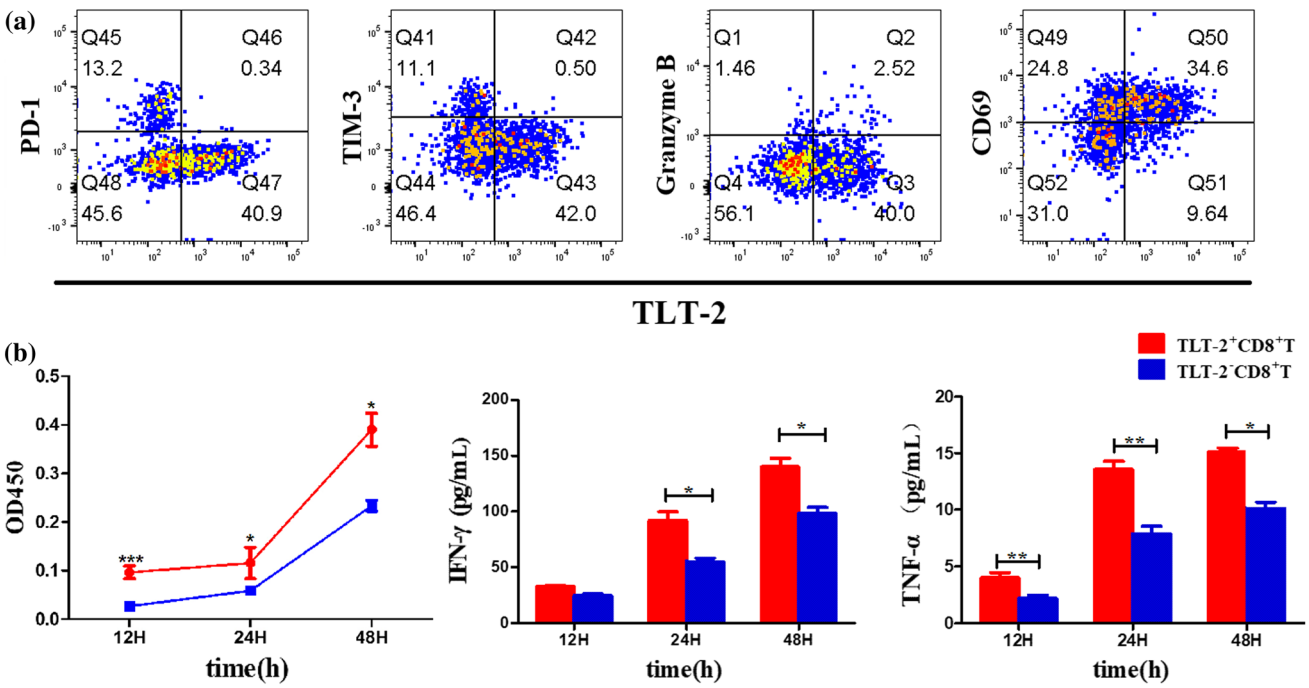

Fig. 2 The differences between the $TLT\text{-}2^+CD8^+T$ and $TLT\text{-}2^-CD8^+T$ cell subgroups in molecular expression, proliferation and cytokine secretion. **a** The relationships between PD-1, TIM-3, CD69, GZMB and TLT-2; samples were isolated from the peripheral blood of acute hepatitis B patients. **b** The analyses of the proliferation and cytokine secretion (IFN-γ and TNF-α) of the $TLT\text{-}2^+CD8^+T$ and $TLT\text{-}2^-CD8^+T$ cell subgroups and the changes with the time of cell culture. *$p<0.05$; **$p<0.01$; ***$p<0.001$

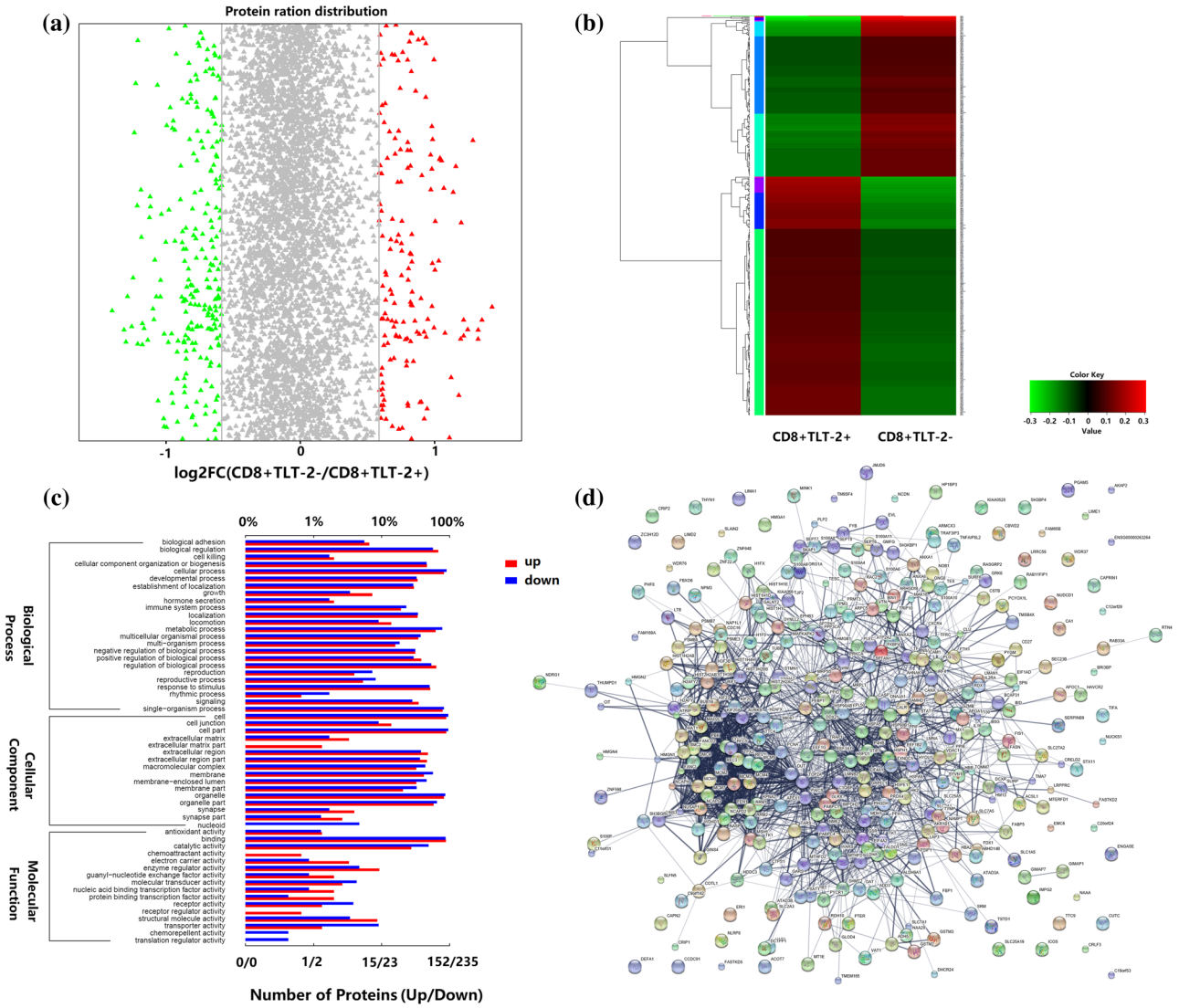

Fig. 3 The iTRAQ and bioinformatics analyses of the proteins that were differentially expressed between the TLT-2$^+$CD8$^+$T and TLT-2$^-$CD8$^+$T cell subgroups. **a** Volcano plot of the proteins expressed in the TLT-2$^-$CD8$^+$T cells compared with the TLT-2$^+$CD8$^+$T cells. The x-axis represents the log2-fold changes in the highly expressed (positive values) and lowly expressed (negative values) proteins in the TLT-2$^-$CD8$^+$T cells compared with the TLT-2$^+$CD8$^+$T cells. Green triangles: lowly expressed proteins in the TLT-2$^-$CD8$^+$T cells compared with the TLT-2$^+$CD8$^+$T cells; red triangles: highly expressed proteins in the TLT-2$^-$CD8$^+$T cell compared with the TLT-2$^+$CD8$^+$T cells. **b** Heatmap of the proteins identified by iTRAQ. **c** GO analysis results showing the up- and down-regulated proteins categorized by biological process, cellular component and molecular function in the TLT-2$^-$CD8$^+$T cells compared with the TLT-2$^+$CD8$^+$T cells. **d** STRING analysis of the interactome network of the proteins that were differentially expressed in the TLT-2$^-$CD8$^+$T cells and TLT-2$^+$CD8$^+$T cells. Each node represents a protein; the large nodes represent proteins with known three-dimensional structures, and the small nodes represent proteins with unknown three-dimensional structures. The lines represent the relationships between the proteins; wider lines indicate stronger correlation coefficient scores

String analysis was used to create an interaction graph of the proteins that were differentially expressed between the two sets of samples and revealed that several proteins, including GZMB, IL-2RA and ICOS, were associated with the activation of CD8$^+$T cells (Fig. 3d).

CD8$^+$TLT-2$^+$T cells participated in CTL anti-HBV infection by regulating the expression levels of GZMB and NK-κB

GZMB, which is now recognized as the major molecule of

the antiviral infection activities of CTLs, was found in 20 of the proteins that exhibited the highest levels of expression differences (5 were up-regulated proteins, and 15 were down-regulated proteins; Table 2). GZMB substrates and the molecules closely related to T cell activation (molecules of the NF-κB signalling pathway, AP-1 signalling pathway and NFAT signalling pathway) were selected from the differential proteins and used to create a volcano plot. BID, which is the substrate of GZMB, was significantly decreased, and the IκBα subunit p100 was significantly increased. According to western blot analysis, these results were similar to those obtained from the mass spectrometry analysis (Fig. 4).

Due to its effects on the NK-κB signalling pathway, TLT-2 mediates the antiviral immunity of CTLs by up-regulating the expression of GZMB

To further investigate the role of TLT-2 in $CD8^+$T cells, shRNA-TLT-2 was used to knock down the TLT-2 expression in normal human $CD8^+$T cells. GFP-positive cells were sorted by flow cytometry. Q-PCR and western blot were used for identification (Fig. 5a, b). Previous studies revealed that the expression of TLT-2 is significantly increased after the activation of $CD8^+$T cells in normal subjects. Compared with the control group, we found that the down-regulation of TLT-2 expression affected the activation of $CD8^+$T cells, decreased the secretions of IFN-γ and TNF-α, and reduced the expression levels of NF-κB (P100) and GZMB.

Discussion

TLT-2 is a member of the TREM family. TLT-2 plays an important regulatory role in innate and adaptive immunity [10–13, 23, 24], and it is the only molecule of the TREM family that is expressed on both T cells and B cells [14] Unlike the high expression levels on other cell surfaces, TLT-2 was expressed at a lower level on naïve $CD8^+$T cells, but this level was significantly increased when the $CD8^+$T cells were activated. Presumably, TLT-2 plays a role in the activation of $CD8^+$T cells. In recent years, research into TLT-2 has received increasing attention. Relationships of TLT-2 with various diseases have been continuously uncovered. However, the signalling pathway that is modulated by TLT-2 has not yet been found, and its effect on the function of $CD8^+$T cells remains unclear.

This study analysed the TLT-2 expression on surfaces of the $CD8^+$T cells of HBV patients. In comparison with the HC group, we found that the proportions of $CD8^+TLT\text{-}2^+$T cells in the peripheral blood were significantly increased in the AHB, CHB and HBV-LC groups, and the differences were statistically significant with the exception of that for the HBV-HCC group. Among the patient groups, pairwise comparisons revealed that the differences were statistically significant between the AHB group and the other three groups but were not significantly different in the CHB versus HBV-LC, CHB versus HBV-HCC and HBV-LC versus HBV-HCC comparisons. These results indicate that different

Table 2 The top 20 differences of protein expression between TLT-2+ and TLT-2− population

Accession	TLT-2+	TLT-2−	FC (TLT-2-/TLT-2+)	log2FC (TLT-2-/TLT-2+)	Regulate
P23381	145.1	54.9	0.378359752	− 1.402169465	Down
P10144	142.6	57.4	0.402524544	− 1.31285134	Down
P04004	142.2	57.8	0.406469761	− 1.298780067	Down
P34897	141.9	58.1	0.40944327	− 1.288264521	Down
P12004	141	59	0.418439716	− 1.256908303	Down
P49736	140.7	59.3	0.421464108	− 1.246518319	Down
Q96AG4	140.4	59.6	0.424501425	− 1.2361587	Down
Q9H967	140.1	59.9	0.427551749	− 1.225829048	Down
Q15758	139.8	60.2	0.430615165	− 1.215528969	Down
P01589	139.7	60.3	0.431639227	− 1.212102114	Down
Q9Y2D5	139.7	60.3	0.431639227	− 1.212102114	Down
Q5T9A4	139	61	0.438848921	− 1.188203735	Down
Q06643	138.9	61.1	0.439884809	− 1.184802314	Down
P00747	138.7	61.3	0.441961067	− 1.178008809	Down
P13995	138.7	61.3	0.441961067	− 1.178008809	Down
P84243	39.2	160.8	4.102040816	2.036341847	Up
Q9BZV3	43.1	156.9	3.64037123	1.864085578	Up
Q16777	44.7	155.3	3.474272931	1.796711093	Up
O00479	46	154	3.347826087	1.743224585	Up
P04083	51.4	148.6	2.891050584	1.531593851	Up

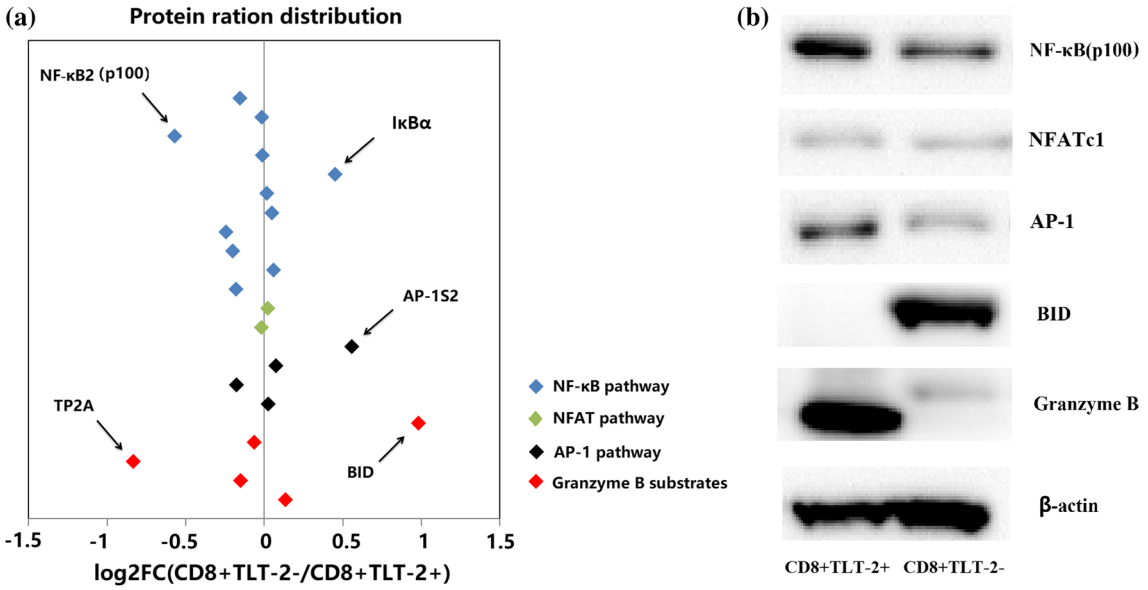

Fig. 4 During HBV infection, TLT-2 affects the function of $CD8^+$T cells by regulating the GZMB and NK-κB signalling pathways. **a** Volcano plot of the special proteins expressed in the $TLT\text{-}2^-CD8^+$T cells compared with the $TLT\text{-}2^+CD8^+$T cells. The x-axis represents the log2-fold changes of the highly expressed (positive values) and lowly expressed (negative values) proteins in the $TLT\text{-}2^-CD8^+$T cells compared with the $TLT\text{-}2^+CD8^+$T cells. Blue squares: the proteins that belong to the NF-κB signal pathway; green squares: the proteins that belong to the NFAT signal pathway; black squares: the proteins that belong to the AP-1 signal pathway; red squares: the substrates of GZMB. **b** Western blot analysis of the differences in GZMB, NF-κB, AP-1, NFAT and BID expression levels between the $TLT\text{-}2^-CD8^+$T and $TLT\text{-}2^+CD8^+$T cells

levels of TLT-2 expression on $CD8^+$T cells may lead to different clinical outcomes. The correlation analysis revealed that TLT-2 expression was positively correlated with ASL and ALT in the AHB and CHB groups. The antigen-specific $CD8^+$T cells are adept at promoting apoptosis of virally infected or transformed cells through delivery of GZMB into target cells via the granule exocytosis pathway [28]. And in our study, we found that increasing the TLT-2 expression on the $CD8^+$T could activate the NF-κB pathway and promote GZMB expression. So when the TLT-2 expression on $CD8^+$T increased, the apoptosis of HBV-infected liver cells caused by HBV antigen-specific $CD8^+$T cell would also increase and thus led to elevated ALT and AST levels in the hepatitis B patients. And combined with the results of our previous study [10], these results further demonstrate that TLT-2 is a very important immune molecule that is closely related to inflammation. Moreover, we found that the expression of TLT-2 on the surfaces of $CD8^+$T cells changed with HBV infection disease progression. Typically, the immune response is competent in adults with acute HBV infection, and the virus is thus completely cleared. In this study, we found that the proportion of $CD8^+TLT\text{-}2^+$T cells was highest in the AHB group among all other groups, which definitely reflected the relationship between the increase in $CD8^+TLT\text{-}2^+$T cells and the robust immune response. Therefore, these results suggest that higher levels of expression of TLT-2 on $CD8^+$T cells might play an important role in the recovery from acute HBV infection. However, the immune response is incompetent in chronically HBV-infected patients; the virus can persist in such hosts for very long periods of time and even result in lifelong infections. The expression level of $CD8^+TLT\text{-}2^+$T cells was lower in the CHB group compared with the AHB group, which is consistent with the outcome of chronic HBV infection. Therefore, the TLT-2 expression in the CHB group may reflect the partial activation of $CD8^+$T cells. In the HBV-LC and HBV-HCC groups, much lower expression levels of TLT-2 on the $CD8^+$T cells were observed, and there were no correlations with the liver enzymes, which indicated that an ineffective immune response occurred in these patients.

The phenotype and cytokine secretions of the $CD8^+TLT\text{-}2^+$T cells were analysed, and the results revealed that the $CD8^+TLT\text{-}2^+$T cells exhibited the characteristics of effect but not exhaust T cells. To investigate the mechanism of TLT-2 in $CD8^+$T cell activation, the proteome analysis and silencing of TLT-2 on $CD8^+$T cell were used. From these two experiments, we found that the level of IκBα which was considered to be the inhibitor complex of NF-κB decreased in the TLT-2 positive cell and the IκBα degradation products p100 was increased which was the key member of NF-κB non-classical activation. And in other two T cell activation signalling pathways AP-1 signalling pathway and NFAT signalling pathway, there were no difference. In the study conducted by Huang et al. [29], the NF-κB binding

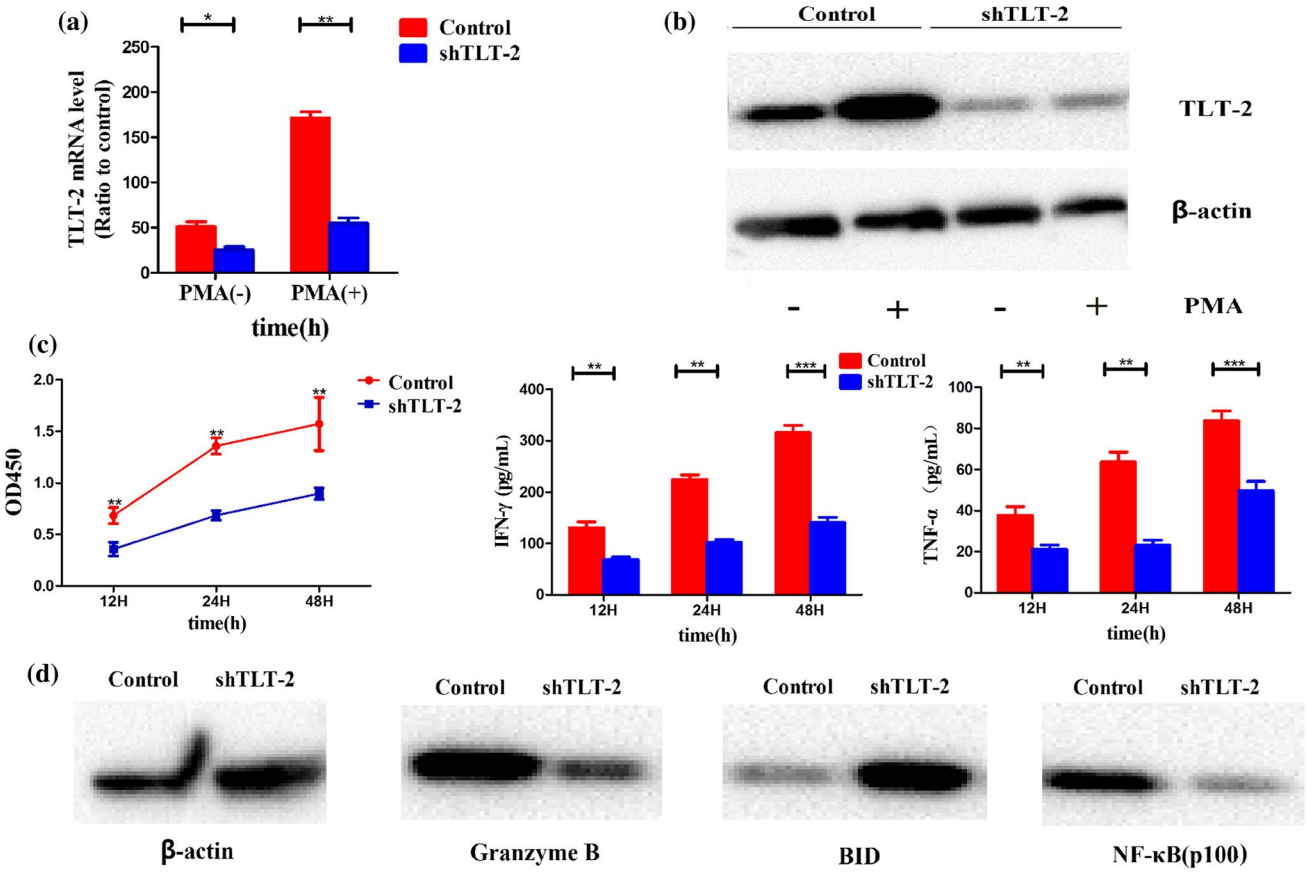

Fig. 5 Knockdown of TLT-2 in human CD8$^+$T cells decreases the sensitivity of CD8$^+$T cells to PMA. **a** The reductions in TLT-2 mRNA with shRNA-mediated knockdown were verified by qRT-PCR. **b** The reductions in TLT-2 protein with shRNA-mediated knockdown were verified by Western blot. **c** The proliferation and IFN-γ and TNF-α levels were significantly decreased in the TLT-2-knocked down CD8$^+$T cells in response to PMA treatment. **d** Western blot analysis of the differences in GZMB, NF-κB and BID expression between the TLT-2-knocked down CD8$^+$T cells and the control cells. $*p<0.05$; $**p<0.01$; $***p<0.001$

site controls human GZMB gene transcription, so we further analysed the expression level of GZMB and found that it increased in the TLT-2 positive cell, while this phenotype disappeared if the expression of TLT-2 was down-regulated. So the TLT-2 can promote the expression of GZMB by NF-κB activation and these are closely associated with the antiviral immune response. The above results demonstrated that the TLT-2 molecule participated in CD8$^+$T cell activation and played an important role in liver inflammation caused by HBV infection.

Conclusion

This is the first study of the role of the effect of TLT-2 on CD8$^+$T cells in infectious disease and proved the close relationship between TLT-2 expression and the clinical outcomes of HBV-infected patients. Based on these results, we believe that the proportion of CD8$^+$TLT-2$^+$T cells in the peripheral blood of patients with HBV infection reflects the host's immune status and is thus closely associated with liver inflammation. Therefore, the detection of TLT-2 expression on the surfaces of T cells in HBV-infected patients is helpful for understanding immune function. Moreover, the present results confirm that TLT-2 regulates the expression of GZMB, which influences the participation of CTLs in antiviral immunity regulating the NF-κB signalling pathway. These results lay a theoretical foundation for the further study of TLT-2. Although our results were carried out in the context of HBV, the TLT-2 seemed to be a molecule that is related to liver damage and inflammation rather than HBV infection. Therefore, our study provides a theoretical basis for a further understanding of the mechanism of hepatitis and the potential treatment value of TLT-2 immune regulation.

Acknowledgements We thank all patients, clinicians and support staff who participated in this study.

Funding This work was supported by Grants from the Science and Technology Plan of Suzhou, China (SYS201369 and SS201657), and the Health Plan of Jiangsu, China (H201652 and H2017068).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Kamei K, Yasuda T, Ueda T, Qiang F, Takeyama Y, Shiozaki H. Role of triggering receptor expressed on myeloid cells-1 in experimental severe acute pancreatitis. J Hepatobiliary Pancreat Sci. 2010;17(3):305–12.
2. Ott JJ, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine. 2012;30(12):2212–9.
3. Nathan C, Ding A. TREM-1: a new regulator of innate immunity in sepsis syndrome. Nat Med. 2001;7(5):530–2.
4. Ormsby T, Schlecker E, Ferdin J, et al. Btk is a positive regulator in the TREM-1/DAP12 signaling pathway. Blood. 2011;118(4):936–45.
5. Bouchon A, Hernández-Munain C, Cella M, Colonna M. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. J Exp Med. 2001;194(8):1111–22.
6. Daws MR, Sullam PM, Niemi EC, Chen TT, Tchao NK, Seaman WE. Pattern recognition by TREM-2: binding of anionic ligands. J Immunol. 2003;171(2):594–9.
7. Washington AV, Schubert RL, Quigley L, et al. A TREM family member, TLT-1, is found exclusively in the alpha-granules of megakaryocytes and platelets. Blood. 2004;104(4):1042–7.
8. Washington AV, Quigley L, McVicar DW. Initial characterization of TREM-like transcript (TLT)-1: a putative inhibitory receptor within the TREM cluster. Blood. 2002;100(10):3822–4.
9. Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. Curr Opin Immunol. 2009;21(1):38–46.
10. Xu JC, Gao F, Fu FQ, et al. Generation and characterization of two novel monoclonal antibodies produced against human TLT-2 molecule. Monoclon Antib Immunodiagn Immunother. 2013;32(3):216–23.
11. King RG, Herrin BR, Justement LB. Trem-like transcript 2 is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. J Immunol. 2006;176(10):6012–21.
12. Kobori H, Hashiguchi M, Piao J, Kato M, Ritprajak P, Azuma M. Enhancement of effector CD8+ T-cell function by tumour-associated B7-H3 and modulation of its counter-receptor triggering receptor expressed on myeloid cell-like transcript 2 at tumour sites. Immunology. 2010;130(3):363–73.
13. Halpert MM, Thomas KA, King RG, Justement LB. TLT2 potentiates neutrophil antibacterial activity and chemotaxis in response to G protein-coupled receptor-mediated signaling. J Immunol. 2011;187(5):2346–55.
14. Hashiguchi M, Kobori H, Ritprajak P, Kamimura Y, Kozono H, Azuma M. Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. Proc Natl Acad Sci USA. 2008;105(30):10495–500.
15. Leitner J, Klauser C, Pickl WF, et al. B7-H3 is a potent inhibitor of human T-cell activation: no evidence for B7-H3 and TREML2 interaction. Eur J Immunol. 2009;39(7):1754–64.
16. Zhang G, Wang J, Kelly J, et al. B7-H3 augments the inflammatory response and is associated with human sepsis. J Immunol. 2010;185(6):3677–84.
17. Prasad DV, Nguyen T, Li Z, et al. Murine B7-H3 is a negative regulator of T cells. J Immunol. 2004;173(4):2500–6.
18. Suh WK, Gajewska BU, Okada H, et al. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. Nat Immunol. 2003;4(9):899–906.
19. Zhang G, Xu Y, Lu X, et al. Diagnosis value of serum B7-H3 expression in non-small cell lung cancer. Lung Cancer. 2009;66(2):245–9.
20. Vigdorovich V, Ramagopal UA, Lázár-Molnár E, et al. Structure and T cell inhibition properties of B7 family member, B7-H3. Structure. 2013;21(5):707–17.
21. Carrasquillo MM, Allen M, Burgess JD, et al. A candidate regulatory variant at the TREM gene cluster associates with decreased Alzheimer's disease risk and increased TREML1 and TREM2 brain gene expression. Alzheimers Dement. 2017;13(6):663–73.
22. Zheng H, Liu CC, Atagi Y, et al. Opposing roles of the triggering receptor expressed on myeloid cells 2 and triggering receptor expressed on myeloid cells-like transcript 2 in microglia activation. Neurobiol Aging. 2016;42:132–41.
23. Ghani M, Sato C, Kakhki EG, et al. Mutation analysis of the MS4A and TREM gene clusters in a case–control Alzheimer's disease data set. Neurobiol Aging. 2016;42:217.e7–13.
24. Benitez BA, Jin SC, Guerreiro R, et al. Missense variant in TREML2 protects against Alzheimer's disease. Neurobiol Aging. 2014;35(6):1510.e19–26.
25. de Freitas A, Banerjee S, Xie N, et al. Identification of TLT2 as an engulfment receptor for apoptotic cells. J Immunol. 2012;188(12):6381–8.
26. Albers I, Hartmann H, Bircher J, Creutzfeldt W. Superiority of the Child–Pugh classification to quantitative liver function tests for assessing prognosis of liver cirrhosis. Scand J Gastroenterol. 1989;24(3):269–76.
27. Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis. 1999;19(3):329–38.
28. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev. 2010;235(1):105–16.
29. Huang C, Bi E, Hu Y, et al. A novel NF-kappaB binding site controls human granzyme B gene transcription. J Immunol. 2006;176(7):4173–81.