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Contribution of IL-17 to Mouse Hepatitis Virus Strain 3-induced Acute Liver Failure*

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Summary: Recently, the Th17 cells and IL-17 have been shown to play a critical role in the immune-mediated liver injury in hepatitis B, while their functions in acute liver failure have not been well elucidated yet. In this study, we primarily investigated the role of IL-17 in the development of mouse hepatitis virus strain 3 (MHV-3)-induced acute liver failure. IL-17 mRNA levels in liver tissue were quantified by using quantitative real-time polymerase chain reaction, and cytokine IL-17 levels in liver tissue and serum were determined by using ELISA in MHV-3-induced murine fulminant hepatitis model. The IL-17 expression levels on $CD4^+$T and $CD8^+$T cells were determined by using flow cytometry. The correlation between IL-17 level and liver injury was studied. Th17 associated cytokines were also investigated by intracellular staining. Our results showed that the IL-17 expression was significantly elevated in the liver and serum of BALB/cJ mice infected with MHV-3. Moreover, a time course study showed that the percentage of both IL-17-producing $CD4^+$T cells and IL-17-producing $CD8^+$T cells was increased remarkably in the liver starting from 48 h and peaked at 72 h post-infection. There was a close correlation between hepatic or serum IL-17 concentration and the severity of liver injury defined by ALT level, respectively. Th17 associated cytokines, IL-6, IL-21 and IL-22, were also increased significantly at 72 h post-infection. It was concluded that IL-17 may contribute to the pathogenesis of MHV-3-induced acute liver failure.
Key words: Th17 cell; IL-17; MHV-3; acute liver failure; virus-induced liver injury

Th17 cells are a more recently discovered subset of $CD4^+$ T-helper cells characterized by the production of cytokine IL-17, playing bidirectional roles in the immune response of inflammation, which are manifested as promoting protective immune responses against a variety of microbes, including several bacterial and fungal pathogens, as well as inducing inflammation, or even leading to autoimmunity[1-3]. Since the liver is a large innate immune organ, Th17 cells are reported to play an important role in the pathogenesis of many liver diseases by regulating innate immunity, adaptive immunity, and autoimmunity[4-6].

Recent studies suggested that Th17 cells and IL-17 play a pivotal role in the immune-mediated liver injury in hepatitis B[7-9]. It was reported that antigen non-specific Th17 response was increased in patients with chronic hepatitis B (CHB) and the peripheral Th17 frequency in these patients was closely associated with severity of liver damage which is determined by serum alanine amino-transferase (ALT) level and liver histological activity index (HAI) score. Moreover, another report also demonstrated that the frequency of Th17 cells was positively correlated with serum ALT levels in CHB patients[10]. However, the regulatory mechanism of Th17 responses in patients with HBV-induced liver failure remains unclear.

In present study, we measured the level of IL-17 in serum and liver tissue from mice with fulminant hepatitis induced by mouse hepatitis virus strain 3 (MHV-3) as well as percentage of IL-17 producing hepatic $CD4^+$T and $CD8^+$T cells to investigate the possible role of IL-17 played in the pathogenesis of virus-induced liver failure.

1 MATERIALS AND METHODS

1.1 Virus

MHV-3 was obtained from the American Type Culture Collection (ATCC, USA). Plaques were purified on monolayer of DBT cells, and titer was tested on L2 cells as previously described[11].

1.2 Animals

Female BALB/cJ mice, aged 6–8 weeks and weighing 18-20 g, were supplied by the Experimental Animal Center of Tongji Hospital (Wuhan, China).

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MHV-3-induced fulminant hepatitis model was established according to the methods of Ning[12]. Mice infected with 100 pfu MHV-3 were used. All animals received care in compliance with the guidelines for the Care and Use of Laboratory Animals and this study was approved by the Committees on Animal Experimentation, Tongji Hospital.

1.3 Total RNA Isolation and Real-time PCR

The isolated livers were dissolved in Trizol (Invitrogen, USA) readily after the harvest. The total RNA was extracted and quantitative real-time PCR was performed. The real-time PCR primers for mouse IL-17A (mIL-17A) and mouse GAPDH (mGAPDH) genes were designed according to the mouse IL-17 sequence (NM_010552) and mouse GAPDH (NM_008084.2), respectively. Details of primers were as follows: mouse IL-17, sense[13], 5′-GGCTGACCCCTAAGAAACC-3′, antisense, 5′-CTGAAAATCAATAGCACGAAC-3′; mGAPDH, sense, 5′-CTCATGACCACAGTCCATGCC ATC-3′, antisense, 5′-CTGCTTCACCACCTTCTTG ATGTC-3′. SYBR Green quantitative PCR Master Mix (Toyobo, Japan) was used in the quantitative PCR tests. All PCRs were performed in a total volume of 20 μL. Relative quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the target gene of each sample and the reference gene GAPDH.

1.4 ELISA

IL-17A in serum and liver tissues was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (Dakewe, Beijing, China) according to the manufacturer's instructions. Assays were performed in duplicate.

1.5 Flow Cytometry

Liver mononuclear cells were separated from parenchymal hepatocytes and cell debris by centrifugation using 40% Percoll. The liver lymphocytes from BALB/cJ mice were stimulated by PMA/ionomycin for 5 h, while cytokine secretion was inhibited with a protein transport inhibitor monensin. After blocking with anti-CD16/32 mAb, cells were stained with anti-CD3, CD4 and CD8 antibodies (Abs) to detect their surface expression, and with anti-IL-17 Ab (Biolegend, USA) to detect their intracellular expression, according to the manufacturer's directions.

1.6 Statistical Analysis

Statistical significance was determined using the Student's *t* test (2-tailed) or ANOVA, as appropriate. The expression of IL-17 in liver/serum correlated with ALT level was confirmed by linear regression analysis. All statistical analyses were performed with SPSS v.11 (SPSS, USA). A *P* value of less than 0.05 was considered statistically significant. All results are presented as $\bar{x}\pm s$.

2 RESULTS

2.1 Elevated IL-17 Expression in Liver and Serum of Fulminant Hepatitis Mice

In MHV-3-infected Balb/cJ mice, compared to the basal expression, the IL-17 mRNA level in liver tissue was increased significantly at 72 h post-infection (90.94%±2.01% *vs.* 1.31%±0.53%, $P<0.001$). Correspondingly, there was a remarkable elevation of IL-17 protein level in the liver at 72 h post-infection measured by using ELISA (139.35±44.78 *vs.* 53.82±21.93 pg/mL, $P=0.009$). Similarly, the IL-17 protein expression was increased conspicuously in serum of infected Balb/cJ mice at 72 h (108.65±13.28 *vs.* 53.65±14.04 pg/mL, $P=0.003$).

2.2 Up-regulated Percentage of IL-17-producing T Cells

A time course study of the percentage of IL-17-producing T cells was analyzed by FACS. Representative flow cytometric plots demonstrated the increase in the populations of IL-17-producing cells when comparing pre- and post-infection samples (fig. 2A). The percentage of IL-17-expressing $CD4^+$T (Th17) cells was increased significantly starting from 48 h (fig. 2B, 2.33%±0.47% at 48 h *vs.* 0.73%±0.06% at 0 h , $P=0.007$), and significantly greater at 72 h than at 48 h post-infeciton (4.43%±0.95% at 72 h *vs.* 2.33%±0.47% at 48 h, $P=0.001$). Meanwhile, the percentage of IL-17-expressing $CD8^+$T cells was also elevated remarkably at 48 h (fig. 2B, 2.87%±1.12% at 48 h *vs.* 1.90%±0.70% at 0 h, $P=0.031$), and peaked at 72 h post-infection (3.97%±0.38% at 72 h *vs.* 1.90%±0.70% at 0 h, $P=0.009$).

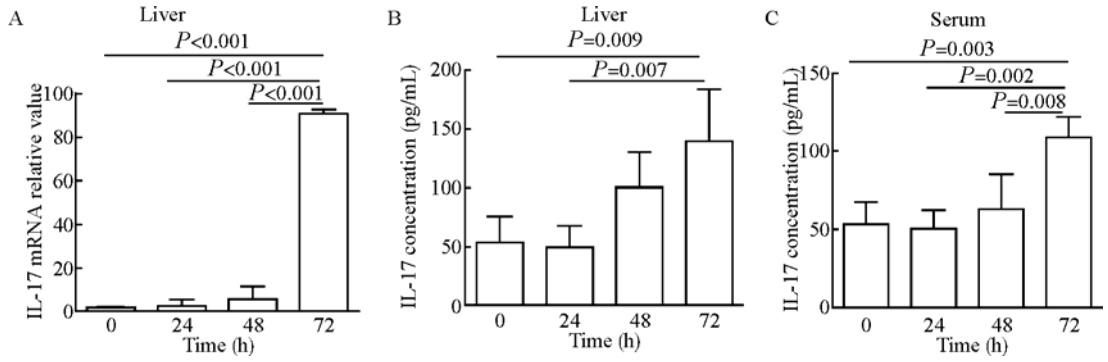

Fig. 1 Elevated IL-17 expression in the liver and serum of fulminant hepatitis mice

After MHV-3 infection, the IL-17 mRNA level in the liver tissue was increased significantly at 72 h compared to the basal expression (A, $P<0.001$). Correspondingly, there was remarkable elevation of IL-17 protein in the liver at 72 h post-infection measured by ELISA (B, $P=0.009$). Similarly, IL-17 protein expression was increased conspicuously in serum of infected BALB/cJ mice at 72 h (C, $P=0.003$)

2.3 Correlation between Increased IL-17 and Severity of Liver Injury in MHV-3-induced Fulminant Hepatitis

The concentration of IL-17 in the liver tissue at 72 h in MHV-3-infected mice was correlated with the corresponding ALT level (fig. 3A, r=0.90, P=0.014). Data also indicated that there was a close correlation between serum IL-17 and the severity of the disease determined by ALT level (fig. 3B, r=0.83, P=0.043). However, there was no significant correlation between the percentage of IL-17-producing CD4$^+$T and ALT level as shown in fig. 3C (r=0.75, P=0.088).

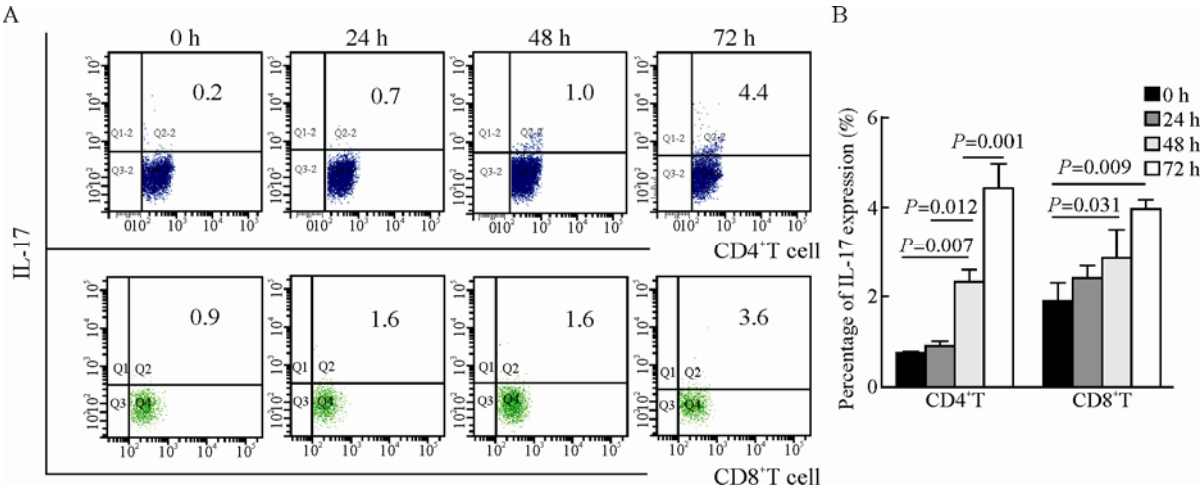

Fig. 2 Up-regulated percentage of IL-17-producing T cells

Representative flow cytometric plots demonstrate the increases in the populations of IL-17-producing T cells after MHV-3 infection (A). The percentage of IL-17-expressing CD4$^+$T (Th17) cells was increased significantly starting from 48 h (B, 48 h *vs.* 0h, P=0.007), and significantly higher at 72 h than at 48 h post-infeciton (B, 72 h *vs.* 48 h, P=0.001). Meanwhile, the percentage of IL-17-expressing CD8$^+$ T cells was also elevated remarkably starting from 48 h (B, 48 h *vs.* 0 h, P=0.031), and peaked at 72 h post-infection (B, 72 h *vs.* 0 h, P=0.009).

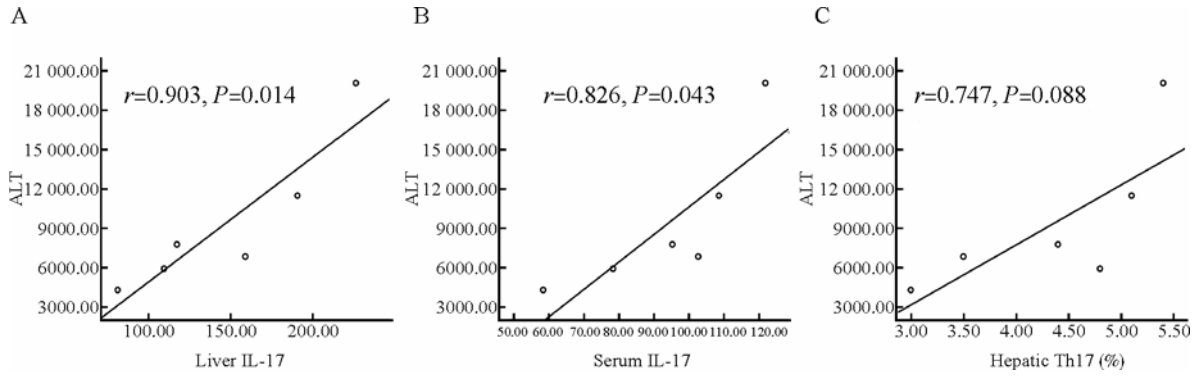

Fig. 3 Correlation between increased IL-17 and the severity of liver injury in MHV-3-induced fulminant hepatitis mice

The hepatic concentration of IL-17 at 72 h in MHV-3-infected mice was correlated with the corresponding ALT level (A, P=0.014). Data also indicated that there was a close correlation between serum IL-17 and the severity of the disease determined by ALT level (B, P=0.043). However, there was no significant correlation between the percentage of Th17 cells and ALT level (C, P=0.088).

2.4 Increased Th17 Associated Cytokines Post-MHV-3 Infection

To further explore the potential mechanisms of increased Th17 contributing to disease progression in murine MHV-3 infection, we examined expression levels of IL-6, IL-21, and IL-22 in hepatic Th17 cells. Analyses of intracellular cytokines staining demonstrated expression levels of IL-6 (1.57%±0.25% *vs.* 0.70%±0.35%, P=0.025), IL-21 (0.37%±0.06% *vs.* 0.13%±0.06%, P=0.008), and IL-22 (0.57%±0.15% *vs.* 0.10%±0.00%, P=0.034) in hepatic Th17 cells at 72 h post-infeciton were significantly higher than that in uninfected mice (fig. 4).

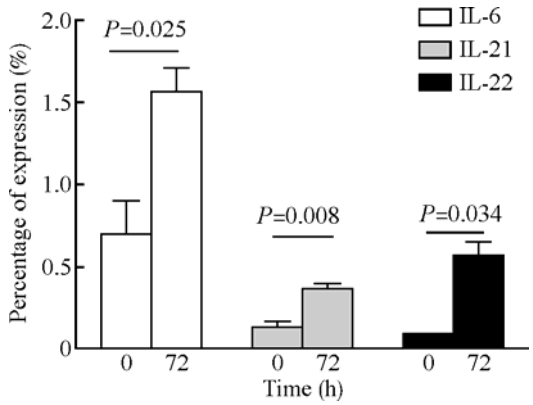

Fig. 4 Increased Th17 associated cytokines after MHV-3 infection

Analyses of intracellular cytokines staining demonstrated the up-regulated expression levels of IL-6, IL-21, and IL-22 in hepatic Th17 cells at 72 h after infeciton were significantly higher than that in uninfected mice.

3 DISCUSSION

The pathogenesis of HBV-induced acute-on-chronic liver failure (HBV-ACLF) remains largely unclear. However, it is generally accepted that immune-mediated mechanisms play a critical role in the pathogenesis. There were many studies providing evidence linking Th17 cells with immune-mediated liver injury. For instance, activated hepatic Th17 cells are responsible for neutrophil recruitment into the liver in alcoholic liver disease[14]. Furthermore, serum levels of IL-17 are increased and served as an evaluable marker for the severity of acute hepatic injury induced by different reasons[15]. And recent studies suggest that Th17 cells play an important role in HBV-induced liver injury, indicating a close correlation between virus-induced liver inflammation and activation of Th17 cells[8-10, 16, 17]. Zhang reported that the peripheral Th17 frequency in CHB patients was closely associated with the degree of liver damage. A shift from Th1 to Th17 seems to be disadvantageous for the patients, since stronger Th17 responses are associated with higher viral plasma load, increased levels of serum transaminases, and enhanced activation of blood monocytes and liver macrophages[8].

There are also reports indicating the role of IL-17 in established animal models of liver injury[18-21]. Reports showed that IL-17-deficient mice developed reduced liver injury compared to wild type mice in Con-A-induced hepatitis[18, 21]. In Halothane-induced liver injury model, plasma levels of IL-17 are increased, and administration of a neutralizing anti-mouse IL-17 antibody leads to decreases in serum AST and ALT levels, while administration of recombinant IL-17 elevates plasma transaminases[22].

However, liver injuries in these models are all induced by chemical agents and viral replication is absent. In order to detect the role of IL-17 in virus-induced liver failure, a MHV-3-induced murine fulminant hepatitis model was introduced. In our study, we confirmed the IL-17 expression was elevated in the liver and serum of fulminant hepatitis mice. Data also indicated that there was a close correlation between hepatic or serum IL-17 concentration and the severity of the disease determined by ALT level, respectively. Moreover, a time-course study showed that the percentage of IL-17-producing $CD4^+$T cells was elevated remarkably at 48 h and peaked at 72 h post-infection, as well as the IL-17-producing $CD8^+$T cells. Our data suggested IL-17 may play an important role in MHV-3-induced liver failure.

Nevertheless, the mechanisms of IL-17 contributing to the development of liver failure remains unclear. During human chronic HBV infection, the increased Th17 cells may further activate mDCs and monocytes to release inflammatory cytokines[8]. While in animal models, it was reported that the increased levels of IL-17 were paralleled with the severity of liver injury as well as the secretion of tumor necrosis factor (TNF)-alpha and IL-6 in Con-A-induced hepatitis. High expression of IL-17R on Kupffer cells was also observed along with the production of cytokines, while inhibition of Kupffer cells completely prevented Con-A-induced liver injury and cytokine release[19]. In current study, we discovered up-regulated expression levels of IL-6, IL-21, and IL-22 in hepatic Th17 cells post-infection, which was consistent with previous findings that activated Th17 cells secrete IL-17A, IL-17F, IL-21 and IL-22, which could promote tissue inflammation by induction of other proinflammatory mediators and recruitment of leukocytes[23-26]. The detailed mechanisms of IL-17 contributing to liver failure in the current model need to be further investigated.

In summary, our study described that the hepatic and serum IL-17 levels, as well as frequency and cytokines secretion of hepatic Th17 cells, were significantly elevated in MHV-3-induced fulminant hepatitis mice. We also showed a close correlation between IL-17 concentration and the severity of liver injury determined by ALT level. These data first discussed the role of IL-17 and Th17 cells in a MHV-3-induced liver failure model, which provided new thoughts into the mechanism of IL-17 involved in HBV-induced liver failure.

REFERENCES

1 Khader SA, Gopal R. IL-17 in protective immunity to intracellular pathogens. Virulence, 2010,1(5):423-427
2 Korn T, Bettelli E, Oukka M, *et al*. IL-17 and Th17 cells. Annu Rev Immunol, 2009,27:485-517
3 Zhao L, Tang Y, You Z, *et al*. Interleukin-17 contributes to the pathogenesis of autoimmune hepatitis through inducing hepatic interleukin-6 expression. PLoS One, 2011,6(4):e18909
4 Hammerich L, Heymann F, Tacke F. Role of IL-17 and Th17 cells in liver diseases. Clin Dev Immunol, 2011,2011:345803
5 Ye C, Li WY, Zheng MH, *et al*. T-helper 17 cell: A distinctive cell in liver diseases. Hepatol Res, 2011,41(1):22-29
6 Rowan AG, Fletcher JM, Ryan EJ, *et al*. Hepatitis C virus-specific Th17 cells are suppressed by virus-induced TGF-beta. J Immunol, 2008,181(7):4485-4494
7 Zhang F, Yao S, Yuan J, *et al*. Elevated IL-6 receptor expression on CD4+ T cells contributes to the increased Th17 responses in patients with chronic hepatitis B. Virol J, 2011,8:270

8 Zhang JY, Zhang Z, Lin F, *et al*. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. Hepatology, 2010,51(1):81-91
9 Wu W, Li J, Chen F, *et al*. Circulating Th17 cells frequency is associated with the disease progression in HBV infected patients. J Gastroenterol Hepatol, 2010,25(4):750-757
10 Ge J, Wang K, Meng QH, *et al*. Implication of Th17 and Th1 cells in patients with chronic active hepatitis B. J Clin Immunol, 2010,30(1):60-67
11 Marsden PA, Ning Q, Fung LS, *et al*. The Fgl2/fibroleukin prothrombinase contributes to immunologically mediated thrombosis in experimental and human viral hepatitis. J Clin Invest, 2003,112(1): 58-66
12 Ning Q, Brown D, Parodo J, *et al*. Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. J Immunol, 1998,160(7):3487-3493
13 Xu S, Han Y, Xu X, *et al*. IL-17A-producing gammadeltaT cells promote CTL responses against Listeria monocytogenes infection by enhancing dendritic cell cross-presentation. J Immunol, 2010,185(10):5879 -5887
14 Lemmers A, Moreno C, Gustot T, *et al*. The interleukin-17 pathway is involved in human alcoholic liver disease. Hepatology, 2009,49(2):646-657
15 Yasumi Y, Takikawa Y, Endo R, *et al*. Interleukin-17 as a new marker of severity of acute hepatic injury. Hepatol Res, 2007,37(4):248-254
16 Zenewicz LA, Yancopoulos GD, Valenzuela DM, *et al*. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. Immunity, 2007,27(4):647-659
17 Ye Y, Xie X, Yu J, *et al*. Involvement of Th17 and Th1 effector responses in patients with hepatitis B. J Clin Immunol, 2010,30(4):546-555
18 Nagata T, McKinley L, Peschon JJ, *et al*. Requirement of IL-17RA in Con A induced hepatitis and negative regulation of IL-17 production in mouse T cells. J Immunol, 2008,181(11):7473-7479
19 Yan S, Wang L, Liu N, *et al*. Critical role of interleukin-17/interleukin-17 receptor axis in mediating Con A-induced hepatitis. Immunol Cell Biol, 2012,90(4):421-428
20 Yu H, Huang J, Liu Y, *et al*. IL-17 contributes to autoimmune hepatitis. J Huazhong Univ Sci Technolog Med Sci, 2010,30(4):443-446
21 Lafdil F, Wang H, Park O, *et al*. Myeloid STAT3 inhibits T cell-mediated hepatitis by regulating T helper 1 cytokine and interleukin-17 production. Gastroenterology, 2009,137(6):2125-2135
22 Kobayashi E, Kobayashi M, Tsuneyama K, *et al*. Halothane-induced liver injury is mediated by interleukin-17 in mice. Toxicol Sci, 2009,111(2):302-310
23 Dong C. Regulation and pro-inflammatory function of interleukin-17 family cytokines. Immunol Rev, 2008,226:80-86
24 Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity, 2008,28(4):454-467
25 Lafdil F, Miller AM, Ki SH, *et al*. Th17 cells and their associated cytokines in liver diseases. Cell Mol Immunol, 2010,7(4):250-254
26 Zhang Y, Cobleigh MA, Lian JQ, *et al*. A proinflammatory role for interleukin-22 in the immune response to hepatitis B virus. Gastroenterology, 2011,141(5):1897-1906

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