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Involvement of CXCR3-associated Chemokines in MHV-3 Induced Fulminant Hepatic Failure*

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Abstract: The role of chemokines in murine hepatitis virus strain 3 (MHV-3) induced fulminant hepatic failure (FHF) is not well defined. In this study, we investigated the role of the CXC chemokine receptor 3 (CXCR3)-associated chemokine [monokine induced by IFN-gamma (Mig/CXCL9) and interferon-gamma-inducible protein 10 (IP-10/CXCL10)] in the recruitment of intrahepatic lymphocytes and subsequent fulminant hepatic failure induced by MHV-3. Balb/cJ mice (6-8 weeks, female) were intraperitioneally injected with 100 PFU MHV-3.The proportions and numbers of T cells and NK cells as well as the expression of CXCR3 on T cells and NK cells in the liver, spleen and blood were analyzed by flow cytometry. The hepatic mRNA level of the CXCR3-associated chemokines (CXCL9 and CXCL10) was detected by realtime PCR. A transwell migration assay was used to assess the chemotactic effect of MHV-3-infected hepatocytes on the splenic lymphocytes. Following MHV-3 infection, the number of hepatic NK cells and T cells and the frequencies of hepatic NK cells and T cells expressing CXCR3 increased markedly; however, in the spleen and peripheral blood, they both decreased significantly. Moreover, the hepatic mRNAs levels of CXCL9 and CXCL10 were significantly elevated post infection. The transwell migration assay demonstrated that MHV-3-infected hepatocytes have the capacity to attract and recruit the splenic NK cells and T cells, and CXCL10 plays a key role in lymphocyte mobilization from the spleen. These results suggest that the CXCR3- associated chemokines (CXCL9 and CXCL10) may play an important role in the recruitment of intrahepatic lymphocytes and subsequent necroinflammation and hepatic failure in MHV-3 infection.

Key words: MHV-3; Liver failure; CXCR3; Chemokine; Flow cytometry

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The pathogenesis of murine hepatitis virus strain 3 (MHV-3)-induced fulminant hepatic failure is a complicated process. Our previous studies demonstrated that the fibrinogenlike protein-2 (Fgl2) prothrombinase expressed by activated macrophages (Kupper cells) plays a crucial role in the pathogenesis of FHF induced by MHV-3 or HBV (3, 7, 8, 13). However, the infiltration of hepatic inflammatory cells should also be noted as another important pathological feature of this disease (5). Substantial accumulated inflammatory cells can not only secrete large amount of inflammatory mediators, causing deterioration of the liver micro-environment, but can also directly induce hepatocyte apoptosis and necrosis. So it is vital to elucidate the role of major chemokines and chemokine receptors involved in this disease, which might even have a potential as therapeutic targets to prevent the development of inflammation and subsequent liver failure. In the present study, MHV-3 induced marked accumulation of hepatic NK cells and T cells with up-regulated expression of CXC chemokine receptor 3 (CXCR3). Furthermore, the expression levels of hepatic CXCR3-associated chemokine (CXCL9 and CXCL10) mRNAs were significantly elevated post infection. Subsequent transwell migration assay demonstrated that infected hepatocytes could attract and recruit the splenic NK cells and T cells, and CXCL10 plays a key role in the recruitment of splenic lymphocytes. These results show that the CXCR3-associated chemokines (CXCL9 and CXCL10) play an important role in the recruitment of intrahepatic lymphocytes and subsequent necroinflammation and hepatic failure in MHV-3 infection.

MATERIALS AND METHODS

Animal model

All animal studies were carried out according to the guidelines of the Chinese Council on Animal Care and approved by the Tongji Hospital of Tongji Medical School Committees on Animal Experimentation. Female Balb/cJ mice (6-8 weeks old, weighing 18-22g) were purchased from the Laboratory Animal Center in Wuhan University (Wuhan, China). All mice were maintained under specific pathogen-free and controlled conditions (22℃, 55% humidity, and 12-h day/ night rhythm) at the Institute of Infectious Disease of Tongji Hospital (Wuhan, China). MHV-3 was obtained from the American Type Culture Collection (ATCC), and plaques purified on monolayers of DBT cells were titered on L2 cells using a standard plaque assay. MHV-3 was reconstituted in sterile phosphate-buffered saline (PBS) at a concentration of 500 PFU/ mL. Mice were injected intraperitoneally with MHV-3 (100 PFU per mouse) in a total volume of 200 μL.

Lymphocyte isolation and quantitation

Under deep ether anesthesia, mice were euthanized by exsanguination from the subclavian artery and vein. A needle was inserted into the portal vein. The liver was perfused with 20 mL pH 7.0 PBS, and then the liver was removed. The liver tissue was thoroughly dissected, gently pressed through a 200-gauge stainless steel mesh, suspended in 45 mL of RPMI 1640 medium containing 10% fetal bovine serum (FBS), centrifuged at 30×*g* for 3 min at room temperature, resuspended, transferred to another 50- mL centrifuge tube, and centrifuged at 500×*g* for 10 min. The pellet was resuspended in 40% Percoll (GE Healthcare, Uppsala, Sweden) solution, layered onto 70% Percoll (GE Healthcare) solution, and centrifuged (800×*g*, 20 min, room temperature). The cells were aspirated from the

Percoll interface, harvested by centrifugation, and washed twice with PBS containing 50 mL/L FBS. The lymphocytes in blood and spleen from Balb/cJ were isolated using NycoPrep™ 1.077A lymphocytes separation medium (AXIS-Shield, Oslo, Norway). All cells were washed and resuspended with PBS containing 0.1% BSA and 0.01% sodium azide [fluorescence activated cell sorting (FACS) buffer]. Cells were counted and stained for cytometry analysis. The total number and percentage of NK ($CD3^-DX5^+$) cells and T ($CD3^+$) cells was calculated as follows: the number of NK cells and T cells in 100 μL of blood was counted by FACS. The total number and percentage of isolated NK cells and T cells in 100 μL of spleen, and liver suspension were determined by FACS. NK cells from liver, spleen were suspended in 500 μL and 5 mL, respectively, of FACS buffer.

Flow cytometric analysis

For phenotyping mouse NK cells and T cells surface markers and analyzing the expression of CXCR3, lymphocytes from the liver, spleen, and blood were stained with the following mAbs: fluorescein isothiocyanate (FITC)-conjugated anti-pan NK cells (DX5), phycoerythrin-CyCrome 5.5(PE-Cy5.5)-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CXCR3, PE rat IgG2a isotype control (eBioscience, San Diego, CA).

Isolation of hepatocytes

Hepatocytes were isolated from livers of BALB/c mice (36 h post MHV-3 infection or non-infection) using a two-step hepatic portal vein perfusion technique. In brief, after the induction of anesthesia with pentobarbital sodium (400 mg/kg ip), the peritoneal cavity was opened, and the liver was perfused *in situ via* the portal vein for 5 min at 37℃ with calcium-free HEPES buffer and for 10 min with HEPES buffer containing 50 mg/100 mL collagenase D and 70 mg/100 mL $CaCl_2$. The perfusion rate was set at 5 mL/min for both solutions. The viability of hepatocytes was more than 90% by the Trypan blue exclusion test. Isolated hepatocytes were supplemented with 10% FBS PRIM 1640 medium.

Real-time PCR assays

Total RNA was extracted from liver tissue at 0 h, 24 h, 48 h, and 72 h post MHV-3 infection using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, the cDNAs were synthesized. The real-time PCR reactions were performed using a SYBR green PCR kit (Biotium) in Roche Sequence Detection System. The sequences of the primers for CXCL9 were 5'-GATCAAACCTGCC TAGATCC-3' (sense) and 5'-GGCTGTGTAGAACA CAGAGT-3' (anti-sense) and yielded a specific 399bp PCR product. The sequences of the primers for CXCL10 were 5'-ACCATGAACCCAAGTGCTGCC GTC-3' (sense) and 5'-GCTTCA CTCCAGTTAAGG AGCCCT-3' (anti-sense) and yielded a specific 312p PCR product. The amplified 215p fragment of GAPDH cDNA was used as an internal control to ensure equal loading and first strand synthesis with forward primer, 5'-CGGATTTGGTCGTATTGGG-3' and reverse primer, 5'-CTCGCTCCTGGAAGATGG-3'. In the PCR reaction the DNA was amplified over 36 cycles, denatured at 94℃ for 40 s, annealed at 60℃ for 45 s, and extended at 72℃ for 60 s. Specificity of the PCR reaction was verified by dissociation-curve analysis and agarose gel electrophoresis.

Transwell migration assays

Transwell migration assays were performed using 6.5 mm transwell inserts (Costar, USA) suspended by

Table 1. Dynamic change of the numbers of NK cells in tissue post MHV-3 infection

Tissue	The number of NK cells			
	0 h	24 h	48 h	72 h
Liver	$1.3\times10^4\pm3.2\times10^3$	$3.4\times10^4\pm1.1\times10^{4*}$	$7.1\times10^4\pm2.7\times10^{4*}$	$4.9\times10^4\pm6.0\times10^{3*}$
Spleen	$6.0\times10^5\pm5.6\times10^4$	$4.3\times10^5\pm4.6\times10^{4*}$	$1.1\times10^5\pm5.8\times10^{4*}$	$7.6\times10^4\pm8.5\times10^{3*}$
Blood	$2.5\times10^4\pm5.5\times10^3$	$1.8\times10^4\pm3.710^{3*}$	$1.0\times10^4\pm2.9\times10^{3*}$	$29.0\pm6.9^*$

Asterisks (*) represent P <0.05 compared with o h group.

the outer rim within individual wells of 24-well ultralow plates (Costar, USA). The wells of the 24-well ultralow plates were filled with 0.6 mL (1×10^4) of normal hepatocyte suspension, 0.6 mL (1×10^4) of MHV-3-infected hepatocyte suspension, 0.6 mL (1×10^4) of MHV-3-infected hepatocyte suspension with anti-CXCL10/IP-10 neutralizing antibodies (R&D), and 0.6 mL PRIM 1640 medium with or without recombinant IP-10 (CXCL10) (PeproTech Inc). 1×10^6 spleen cells were added in 100 μL to the top transwell, which was placed in contact with the medium in the wells of the 24-well plates. The migration assay plates were placed in a humidified incubator (37℃, 5% CO_2) for 4 h. After 4 h, the transwells were removed, medium and cells from the lower wells were harvested, and the wells were rinsed once with FACS buffer. The total number of migrated cells was confirmed by microscope count. The proportion of NK cells and T cells was determined by cell staining and FACS assay, and then the total numbers of migrated NK cells and T cells were calculated.

Statistical Analysis

Statistical analysis was performed using Student t test or one-way analysis of variance. Significance is defined by P<0.05.

RESULTS

The dynamic change of the number of NK cells and T cells post infection

Before MHV-3 infection, the number of NK cells and T cells in the liver (1.34×10^4 and 4.29×10^4), spleen (6.01×10^5 and 2.45×10^6), and blood (2.53×10^4 and 3.15×10^5) were measured using FACS analysis. As shown in Table 1 and Table 2, post MHV-3 infection, the numbers of NK cells and T cells in the liver were both markedly increased, while their number in the spleen and blood were both significantly reduced. 48h post infection, the numbers of NK cells and T cells in the liver increased to 7.11×10^4 and 7.73×10^4, respectively. In parallel, the number of NK cells and T cells in spleen were reduced to 1.08×10^5 and 4.08×10^5. In blood, the numbers of NK cells and T cells were also reduced to 1.01×10^4 and 2.80×10^4. These results suggest that NK cells and T cells may migrate to liver from blood and spleen post MHV-3 infection.

Table 2. Dynamic change of the numbers of T cells in tissue post MHV-3 infection

Tissue	The number of T cells			
	0 h	24 h	48 h	72 h
Liver	$4.3\times10^4\pm7.7\times10^3$	$5.4\times10^4\pm1.0\times10^{4*}$	$7.7\times10^4\pm4.0\times10^{4*}$	$7.6\times10^4\pm7.2\times10^{4*}$
Spleen	$2.5\times10^6\pm1.1\times10^6$	$1.4\times10^5\pm4.6\times10^{4*}$	$4.1\times10^5\pm1.9\times10^{5*}$	$4.5\times10^5\pm3.1\times10^{5*}$
Blood	$3.2\times10^5\pm6.8\times10^4$	$1.2\times10^5\pm2.4\times10^{4*}$	$2.8\times10^4\pm8.1\times10^{3*}$	$1731.3\pm413.6^*$

Asterisks (*) represent P <0.05 compared with o h group.

The expression of CXCR3 on NK cells and T cells post infection

At 48 h Post MHV-3 infection, the frequency of hepatic NK cells and T cells expressing CXCR3 were 7.4% and 20.1%, respectively, which were significantly higher than that of the normal control (1.8% and 8.0%). At the same time, the frequency of NK cells and T cells expressing CXCR3 in spleen and blood were markedly reduced from 11.6% and 24.8% to 3.6% and 9.3%, 14.5% and 14.9% to 5.0% and 2.4%, respectively (Fig. 1).

The dynamic change of the hepatic mRNA lever of CXCL9 and CXCL10 post infection

Through real-time PCR, we observed the dynamic change of the mRNA level of CXCR3-associated chemokines (CXCL9 and CXCL10) post MHV-3 infection. We found that, post MHV-3 infection, the mRNA level of hepatic CXCL9 was continuously enhanced, and peaked at 48h post-infection, when it was about 15.6 times the levels of the normal control, and then came down slightly (Fig. 2A). Unexpectedly, the mRNA level of CXCL10 in the liver increased

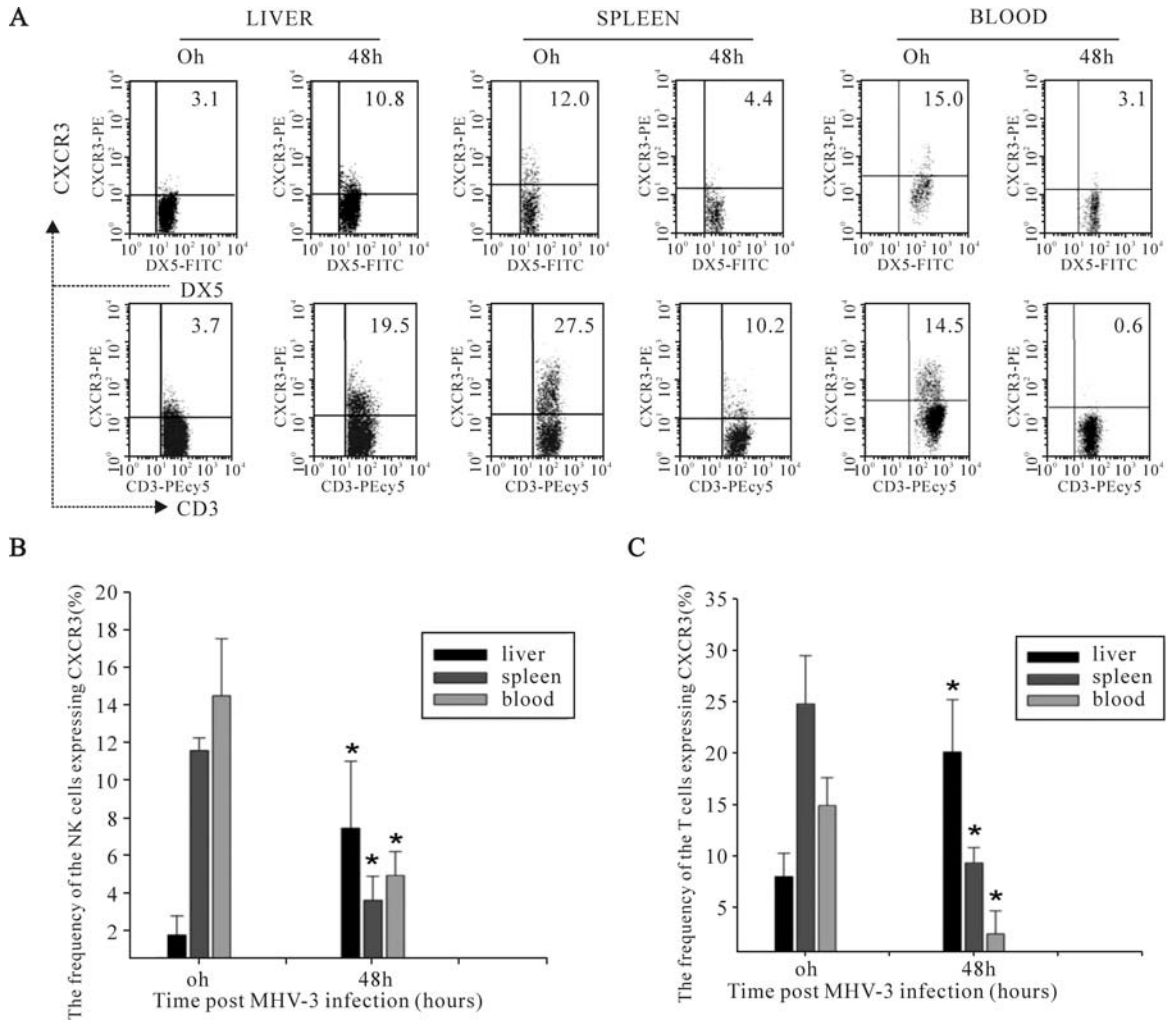

Fig. 1. The expression of CXCR3 on hepatic, splenic , and peripheral T cells and NK cells post MHV-3 infection. A: A representative FACS analysis of the expression of CXCR3 on liver, splenic, and peripheral NK cells ($CD3^-DX5^+$) and T cells ($CD3^+$) post MHV-3 infection. B: Statistical analyses of the frequencies of hepatic, splenic , and peripheral NK cells and T cell expressing CXCR3 post MHV-3 infection. Asterisks (*) represent P <0.05 compared with o h group.

dramatically post infection. Compared with the normal control, the mRNA level of CXCL10 was increased 99x and 150x at 48 h and 72 h post infection, respectively (Fig.2B).

The chemotactic effect of MHV-3-infected hepatocytes on splenic lymphocytes

To further investigate the chemotactic effect of MHV-3-infected hepatocytes on splenic lymphocytes and the role of CXCL10 in the mobilization of splenic lymphocytes, we designed a transwell migration test in vitro. As shown in Fig.3, MHV-3-infected hepatocytes could attract and recruit the splenic NK cells and T cells, and this effect can be blocked effectively by anti-IP10 monoclonal antibody. In addition, CXCL10 protein can also attract the splenic NK cells and T cells. These results suggest a crucial

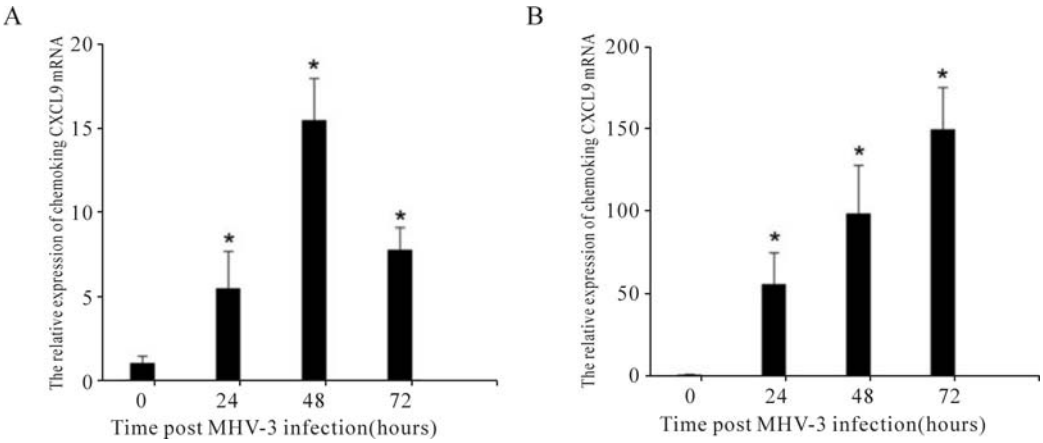

Fig. 2. Dynamic change of the mRNA levels of CXCL9 and CXCL10 in the liver post MHV-3 infection. A: The relative expression of CXCL9 mRNA in liver post infection. B: The relative expression of CXCL10 mRNA in liver post infection. Asterisks (*) represent P <0.05 compared with o h group.

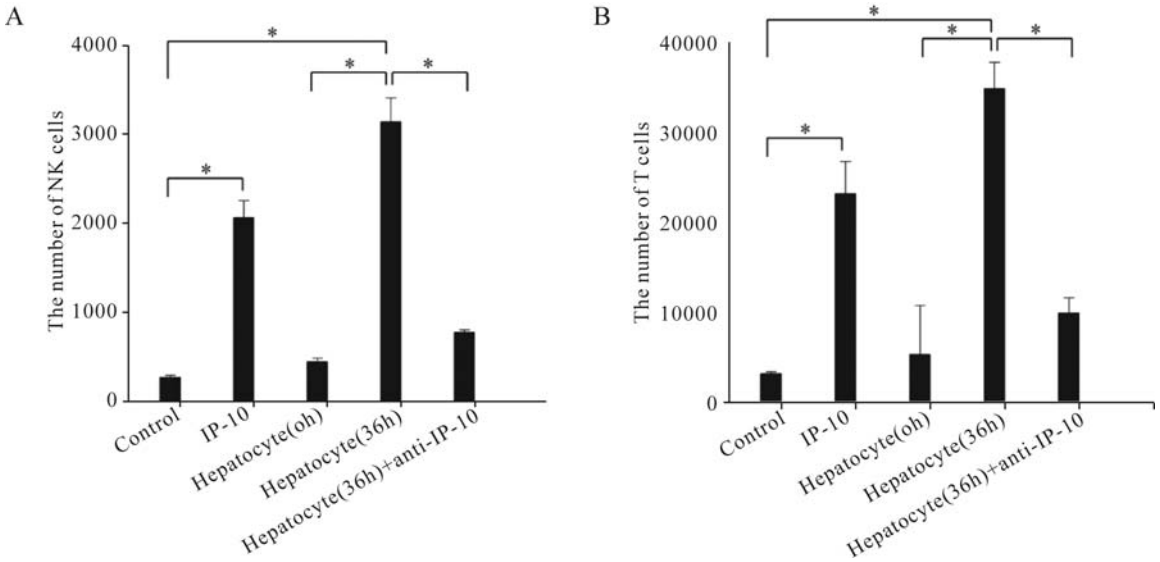

Fig.3. The transwell migration assays. A: The chemotactic effect of MHV-3-infected hepatocytes and IP-10/CXCL10 protein on splenic NK cells. B: The chemotactic effect of MHV-3-infected hepatocytes and IP-10/CXCL10 protein on splenic T cells. Asterisks (*) represent P <0.05 compared with control group.

involvement of CXCL10 in the lymphocytes mobilization from the spleen.

DISCUSSION

In recent years, the role of chemokines in viral hepatitis has been the subject of much scrutiny. Accumulated evidence has demonstrated that the disordered expression of chemokines was closely related with the developmental progress of viral hepatitis (1, 9). The highly up-regulated chemokines in liver recruit large amount of effector and inflammatory cells, resulting in a hypernomic inflammatory reaction and aggravation of hepatocyte injury. So it is vital to clarity the role of chemokines in viral hepatitis, which will help to find new molecular targets for the intervention of clinical treatments.

In a mice model of fulminant hepatic failure (FHF) induced MHV-3, we first found MHV-3 infection recruits a large number of NK and T cells into the liver. To further investigate the mechanism of lymphocytes migration into the liver post infection, we analyzed the expression of correlative chemokines and their receptors. We found that, post MHV-3 infection, the expression frequency of CXCR3 on hepatic NK cells and T cells was significantly increased. Conversely, in the spleen and peripheral blood, the expression frequencies were both significantly decreased. Meanwhile, the mRNA level of the CXCR3-associated chemokines, especially the CXCL10, presented dramatic up-regulation. These results suggested the pivotal involvement of the CXCR3-associated chemokine (CXCL9 and CXCL10) in the recruitment of intrahepatic lymphocytes and subsequent hepatic failure in MHV-3 infection.

Monokines induced by interferon-γ (Mig/CXCL9) and the interfereon-γ-inducible 10-kDa protein (IP-10/CXCL10), which belong to the CXC chemokine family, have strong chemotaxis to T cells, NK cells, mononuclear macrophages and dendritic cells, and play a crucial role in lymphocytes migration, physiological homing and pathological recruitment. CXCL9 and CXCL10 can specifically bind with the chemokine receptor-CXCR3 expressed on the surface of NK and T cells and traffic these cells to a local focus in the liver, which expands local inflammatory reactions and accelerates liver injury. Therefore, the disordered expression of chemokines may have a close association with the development of HBV or HBV-induced liver failure.

Emerging evidence indicates that HBV infection could induce high expression of CXCL10 in HBV-infected hepatocytes and vascular endothelial cells (12). In addition, CXCL10 was also highly expressed in peripheral blood and liver tissue of patients with CHB and HBV-ACLF (2, 4, 10, 11). Finally, the high expression of CXCL10 has a close association with liver necrotizing inflammation and hepatic fibrosis (6). These studies mentioned above suggest that CXCL10 may play a key role in disease progression and severity of HBV and HCV infection. In this study, we also observed a dramatically elevated mRNA level of CXCL10 in the liver tissue and up-regulated expression of CXCR3 on hepatic NK and T cells post MHV-3 infection, which suggests a crucial involvement of the interaction between CXCL10 and its receptor CXCR3 in lymphocytes migration, necroinflammation and the hepatocyte injury in MHV-3-induced FHF. Therefore, further studies of the precise mechanism will help to prevent the development of inflammation and immune-induced liver injury at a molecular level.

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