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Tumor necrosis factor α (TNF- α) receptor-I is required for TNF- α -mediated fulminant virus hepatitis caused by murine hepatitis virus strain-3 infection



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ARTICLE INFO

Article history:

Received 20 September 2013

Received in revised form 5 November 2013

Accepted 5 November 2013

Available online 25 November 2013

Keywords:

Murine hepatitis virus strain-3

TNFR1

FGL2

Neutrophils

ABSTRACT

TNF- α plays an essential role in the pathogenesis of fulminant virus hepatitis (FH) caused by infection with murine hepatitis virus strain-3 (MHV-3). However, the specific TNF- α receptors (TNFR) involved in this disease and how they mediate this effect are uncertain. Here, we showed that the expression of TNFR1 and TNFR2 in the liver and spleen was triggered by MHV-3. However, only TNFR1^{-/-} mice were resistant to MHV-3 mediated FH, as displayed by a dramatic decrease in tissue necrosis and cell apoptosis in the infected spleens and livers from TNFR1^{-/-} mice, as well as prolonged survival in these mice compared to wild type littermate controls. Mechanistically, TNFR1 deficiency directly impeded the serum and tissue levels of fibrinogen-like protein 2 (FGL2), a virus-induced procoagulant molecule that promotes cell apoptosis. Additionally, the expression of apoptosis-associated molecules, Fas and Fas ligand (FasL) in the infected organs from TNFR1^{-/-} mice were also decreased. Moreover, the infiltration of neutrophils rather than Foxp3⁺ regulatory T cells, which produce proinflammatory factors and FGL2 directly, into the infected liver and spleen tissues was also decreased in TNFR1^{-/-} mice. These combined results indicate that signaling through TNFR1 plays an essential role in the pathogenesis of FH caused by MHV-3 infection, and interruption of this signaling pathway could be useful for clinical therapy.

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1. Introduction

Fulminant viral hepatitis (FH) is a clinical syndrome characterized by massive hepatocyte necrosis and hepatic encephalopathy during viral infection [1]. In many Asian countries, including China, where viruses such as hepatitis B are endemic, FH is one of the most common diseases requiring immediate hospitalization. However, historically, it has been difficult to analyze the pathogenesis of FH due to a lack of available animal models. Fortunately, recent studies using a mouse FH model infected by murine hepatitis virus-3 (MHV-3) has provided significant insights into the mechanisms underlying the pathogenesis of this disease and has led to the development of some novel therapeutics [1].

MHV-3 is a single-stranded, positive-sense RNA virus that belongs to the coronavirus family. In susceptible inbred mouse strains such as Balb/cj or C57BL/6, mice infected with MHV-3

develop severe hepatitis and die within 3–7 days, whereas A/J mice are resistant and develop no clinical signs of hepatitis [2]. The hallmark of MHV-3-induced FH in susceptible hosts is the presence of sinusoidal thrombosis and hepatocellular necrosis in conjunction with the expression of a virus-induced procoagulant molecule, fibrinogen-like protein 2 (FGL2), by monocytes/macrophages. Tissue deposition of FGL2 can directly activate a coagulation cascade, which is a phenomenon known as ‘virus-induced procoagulant activity’ (PCA) [3]. Interestingly, blocking FGL2 ameliorates MHV-3-induced liver injury, and fgl2 mutant mice display natural resistance to infection, suggesting that fgl2 plays an essential role in the pathogenesis of FH [4,5].

TNF- α is a pleiotropic cytokine, and up-regulation of TNF- α expression is observed in most inflammatory responses. The biological functions of TNF- α are mediated by two functionally distinct receptors, p55 TNFR1 (TNFRSF1A) and p75 TNFR2 (TNFRSF1B) [6]. TNFR1 is the primary signaling receptor on most cell types and accounts for the proinflammatory, cytotoxic, and apoptotic effects due to the presence of a death domain in its cytoplasmic tail [7]. In contrast, TNFR2 lacks an intracellular death domain and predominantly mediates signals promoting cell proliferation [8].

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Previous works from our group and others have shown that TNF- α -mediated hepatocyte apoptosis is responsible for the pathogenesis of FH caused by MHV-3. Moreover, TNF- $\alpha^{-/-}$ mice were resistant to MHV-3 mediated FH [9–11]. However, which TNFRs are involved in this effect, and how they regulate the pathogenesis of FH are still uncertain.

Here we examined the exact role of TNFR1 and TNFR2 in the disease progression of a mouse FH model caused by MHV-3 infection *in vivo*. Our results showed that a deficiency of TNFR1, rather than TNFR2, signals impeded tissue damage and cell apoptosis by reducing Fas and FGL2 expression following infection. Moreover, tissue infiltration of neutrophils, which can produce FGL2 directly, was also reduced in the TNFR1 $^{-/-}$ mice. These results suggest that TNFR1 plays an essential role in the pathogenesis of FH caused by MHV-3.

2. Materials and methods

2.1. Virus

MHV-3 was kindly provided by Professor Q. Ning (Institute of Infectious Disease, Tongji Hospital of Tongji Medical College, Wuhan, China). The virus was first plaque-purified and then expanded in murine 17CL1 cells to a concentration of 1×10^7 PFU/ml. The virus-containing supernatants were collected and stored at -80°C until use.

2.2. Mice and infections

The TNF- $\alpha^{-/-}$, TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ mice (background C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice were maintained in micro-isolator cages, fed as standard laboratory chow diet and water, and housed in the animal colony at the animal center of The Third Military Medical University. Mice of ~ 12 weeks of age were used for experiments. All of the *in vivo* experiments comply with the animal study protocol approved by the ethics committee of the Third Military Medical University.

Mice were infected with 100 PFU MHV-3 *via* i.p. injection. The survival of virus infected mice was monitored for 20 days at least and the survival curves were generated by the log-rank test, or mice were killed on the indicated days, and the tissues were harvested. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using an Olympus AU5400 automatic biochemistry analyzer, and serum FGL2 and cytokine levels were measured by ELISA as described previously [9]. The liver tissues were harvested, and the morphology was detected by Hematoxylin and Eosin (H&E) staining. No liver damage and fibrinogen deposition was observed in the gene deletion mice without MHV-3 infection (data not shown).

2.3. Immunohistochemical and immunofluorescence staining

Paraffin-embedded tissue blocks were cut into 2–4 μm slices and were mounted onto poly-lysine-charged glass slides. Some sections were used to analyze MHV-3-induced tissue damage by H&E staining. Other tissue sections were used to detect FGL2, fibrinogen, Fas, FasL and TNFR1 expression by immunohistochemistry as described in our previous work [9]. For the immunofluorescence staining, the sections were dewaxed and rehydrated, and antigen retrieval was performed by microwaving the sections in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by exposure to 3.0% H_2O_2 for 30 min. Sections were then incubated at 4°C overnight with antibodies against the following proteins: FGL2 (1:100 dilution; Santa Cruz, Santa Cruz, California, USA), TNFR1 (1:50 dilution; Santa Cruz), TNFR2 (1:50 dilution; Santa Cruz), IFN- γ (1:50 dilution; eBioscience, San Diego, California, USA), TNF- α (1:100 dilution; Santa Cruz), Foxp3 (1:50 dilution; eBioscience), or Gr-1 (1:100 dilution; Santa Cruz). Sections were further treated with 4',6-diamidino-2-phenylindole (DAPI) for an additional 10 min. Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated (1:100 dilution; Zymed Laboratories, San Francisco, California, USA) or Cy3-conjugated secondary antibodies (1:200 dilution; Jackson ImmunoResearch, West Grove, Pennsylvania, USA). The results were analyzed using fluorescence microscopy (Axioplan 2; Zeiss, Oberkochen Germany).

The number of Foxp3 $^+$ -cells in the infected livers and spleens were counted by image analysis of histological sections. Five sections of each sample were randomly selected, and photomicrographs were obtained in high-power fields (0.625 mm 2) and captured for analysis using ImagePro-Plus 5.0 software (Media Cybernetics, SilverSpring, MD, USA). The number of Foxp3 $^+$ -positive cells per high-power field were counted and expressed as the mean \pm standard error of the mean.

2.4. ELISA

Serum TNF- α and FGL2 levels from MHV-3-infected or uninfected mice were determined using the mouse TNF- α and FGL2 ELISA kit, respectively, according to the manufacturer's instructions (Uscn Life Science, Wuhan, China). The protocol has been described previously [9].

2.5. Western-blot

Mice were infected with or without MHV-3, the livers and spleens were collected after 72 h of infection, and the expression of FGL2, TNF- α , TNFR1 and TNFR2 in these organs was detected by western-blot, as described previously [10].

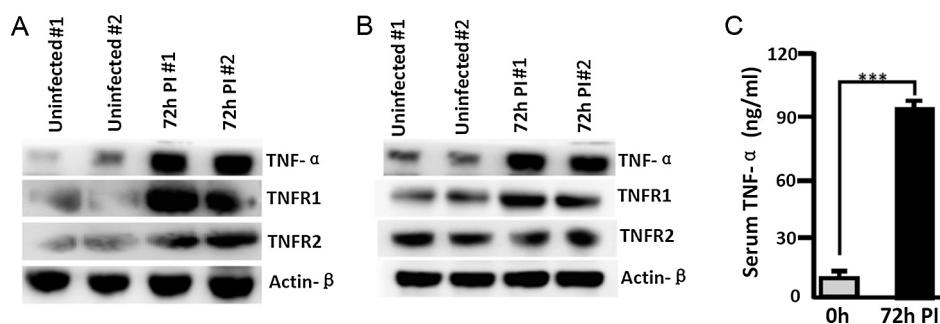


Fig. 1. The expression of TNF- α and its receptors in livers and spleens was triggered by MHV-3. Wild-type (WT) mice on C57BL/6 background were infected with or without MHV-3 (100 PFU), and the expression of TNF- α and its ligands, TNFR1 and TNFR2, in (A) the livers and (B) the spleens was compared by western-blot. Data were from two of represent five mice of each group, PI indicated post infection. (C) Serum TNF- α levels were detected by ELISA. $N = 8-10$ of each group. *** $p < 0.0001$.

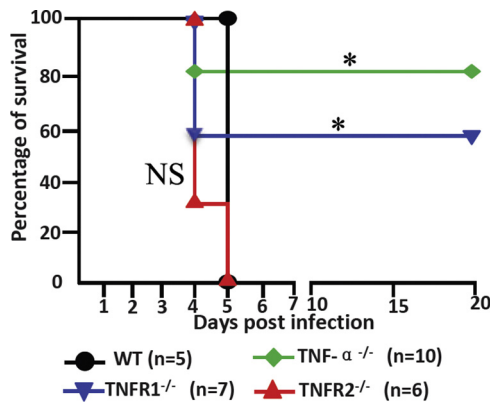


Fig. 2. TNFR1^{-/-} mice resistant to MHV-3 mediated mortality. TNF- α ^{-/-}, TNFR1^{-/-} and TNFR2^{-/-} mice as well as wild-type (WT) littermates (strain C57BL/6) were infected with MHV-3 (100 PFU), and the survival rate was monitored for a total of 20 days. One representative of three experiments with similar results was shown. *N* indicated the number of animal of each experimental group. **p* < 0.05 and NS: not significant different.

2.6. Flow cytometry

Mice were infected with or without MHV-3, the livers and spleens were collected at the indicated time. The infiltration of neutrophils (Gr-1⁺CD45⁺) and CD4⁺Foxp3⁺ regulatory T cells (Treg) into livers and spleens was measured by flow cytometry (FACSAria Cytometer; Becton Dickinson, Franklin Lakes, NJ, USA). Briefly, tissues were grinded and suspended cells were centrifuged. Cells were collected and incubated for 30 min at RT in the dark using fluorescent antibodies. To detect intracellular foxp3 expression, cells

were treated with brefeldin A for 4 h. Thereafter, the fluorescent labeled monoclonal antibodies (mAbs) were then added, and cells were incubated for an additional 1 h. The cells were washed with phosphate-buffered saline, and 10,000 cells were acquired. Flow cytometric data were analyzed using CellQuest Pro software.

2.7. Statistical analyses

All data were analyzed using GraphPad Prism 4.03 software. An unpaired Student *t*-test (two-tailed) was used to assess comparisons between two groups when the data met the assumptions of a *t*-test. Survival curves were generated by the log-rank test. A *p*-value < 0.05 was considered significant. All results shown are representative of at least three separate experiments.

3. Results

3.1. The expression of TNF- α and its receptors is triggered by MHV-3

Virus-sensitive C57BL/6 mice were infected with 100 plaque-forming units (PFU) of the MHV-3 virus *via* i.p. injection. The expression of TNF- α and its ligands, TNFR1 and TNFR2, in the infected organs was analyzed thereafter. Interestingly, the expression of TNF- α and TNFR1 in the infected livers and spleens was increased significantly following MHV-3 infection, as detected by western-blot (Fig. 1A and B). The expression of TNFR2, conversely, was also triggered by MHV-3 in the infected liver but not in the spleen (Fig. 1A and B). As compared to uninfected mice, higher levels of serum TNF- α in the infected mice was also observed (Fig. 1C). The expression of these molecules in other organs including kidney,

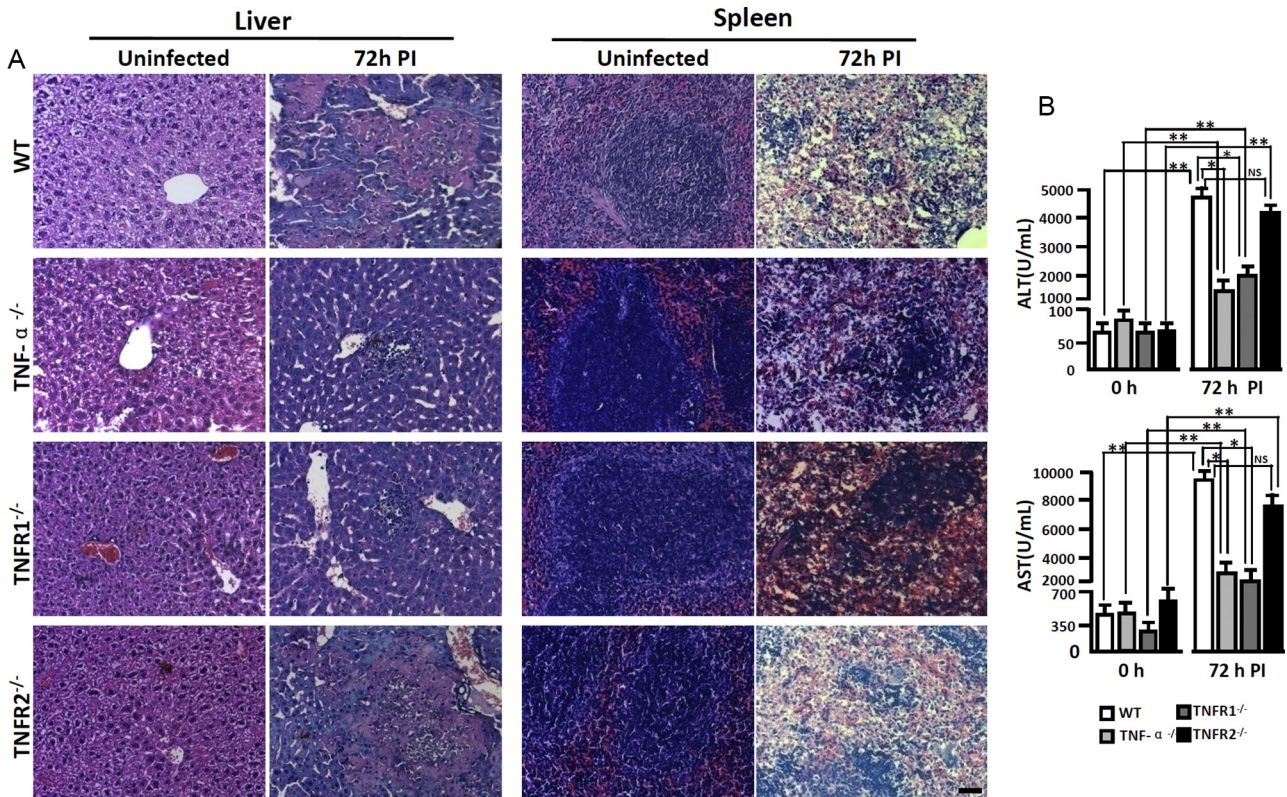


Fig. 3. TNFR1^{-/-} mice resistant to liver and spleen damage caused by MHV-3 infection. (A) TNF- α ^{-/-}, TNFR1^{-/-} and TNFR2^{-/-} mice as well as wild-type (WT) littermates (strain C57BL/6) were infected with MHV-3 (100 PFU), and the architecture of the livers was analyzed by H&E staining. (B) Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined with an AU5400 automatic biochemistry analyzer (*n* = 6–10 per group). Scale bar = 20 μ m.

thymus, heart and lung, was also detected. However, their levels were not changed dramatically (data not shown). These results suggest that the expression of TNF- α and its receptors, in the infected livers and spleens, were triggered by MHV-3.

3.2. TNFR1^{-/-} mice are resistant to MHV-3-mediated tissue damage and mortality

To determine the potential role of TNFR signals in the pathogenesis of FH, TNFR1^{-/-}, TNFR2^{-/-} mice, and WT littermate controls from the virus-sensitive C57BL/6 background were further infected with MHV-3. Surprisingly, all of the infected WT and TNFR2^{-/-} mice were dead within 5 days, while 57% of the TNFR1^{-/-} mice still survived for the full 20 days (Fig. 2). Although no liver and spleen tissue damage was observed in these mice under uninfected conditions, severe necrosis with sparse polymorphonuclear leukocyte infiltration was observed in the WT and TNFR2^{-/-} livers and spleens at 72 h of virus infection, as detected by H&E staining. In contrast, the damaged area of the infected TNFR1^{-/-} mice was less than WT mice under the same conditions (Fig. 3A). In agreement with these observations, TNFR1^{-/-} mice exhibited lower serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) compared with the

levels observed in serum from the infected WT mice (Fig. 3B). The parameters from the infected TNF- α ^{-/-} mice, which have reduced tissue necrosis and prolonged survival time following MHV-3 infection [9], were used for controls. Additionally, no tissue damage was observed in other organs including kidney, thymus, heart and lung in these mice in response to MHV-3 infection (data not shown). These results clearly show that a deficiency of TNFR1-mediated TNF- α signal reduced MHV-3 mediated liver and spleen damage, and thus rendered these mice resistant to MHV-3-mediated mortality.

3.3. Reduced FGL2 secretion in TNFR1^{-/-} mice following MHV-3 infection

Fgl2 is a critical molecule that mediates hepatocellular necrosis and lethality following MHV-3 infection [4,5]. To determine whether fgl2 is involved in the pathogenesis of FH in these mice, we measured the expression of FGL2. First, TNF- α , and TNFR1 were co-expressed with FGL2 on the same cells, as detected by immunofluorescent double staining (Fig. 4A), suggesting that the TNF- α /TNFR1 signal could regulate FGL2 secretion directly. Interestingly, the concentration of FGL2 in the infected livers and spleens from the TNFR1^{-/-} mice was much lower compared to WT mice,

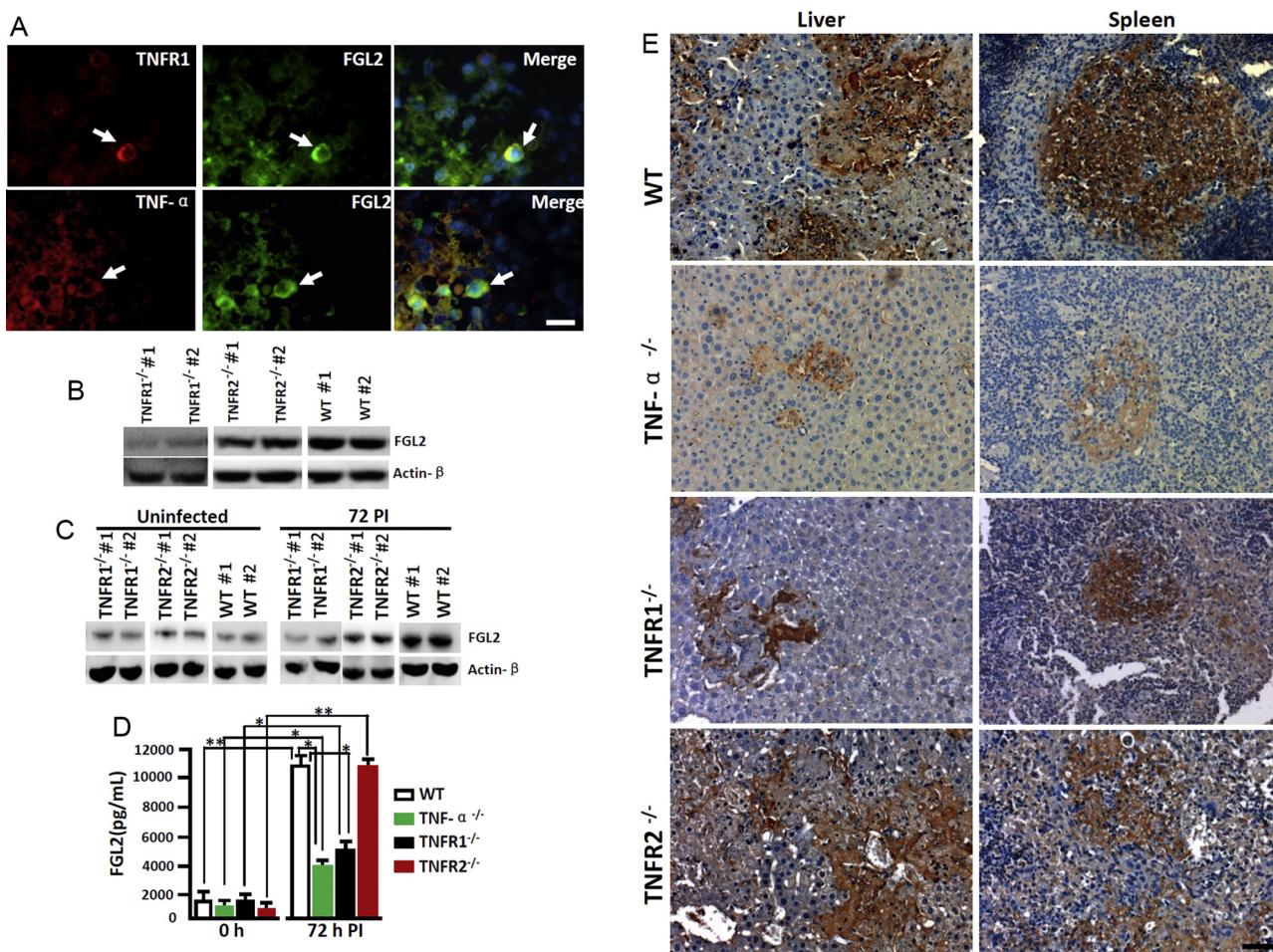


Fig. 4. Reduced FGL2 secretion in TNFR1^{-/-} mice following MHV-3 infection. (A) The expression of TNF- α , TNFR1 and FGL2 was detected by immunofluorescent double staining. Scale bar 20 μ m. Blue color indicates nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). (B) The concentration of FGL2 in livers from MHV-3 infected TNFR1^{-/-}, TNFR2^{-/-} and WT mice was analyzed by Western-blot. Data were from two of represent five mice of each group, PI indicated post infection. (C) The concentration of FGL2 in spleens from TNFR1^{-/-}, TNFR2^{-/-} and WT mice with or without MHV-3 infection was analyzed by Western-blot. (D) The serum FGL2 concentration from infected mice was measured by ELISA ($n = 6-10$ per group). (E) The fibrinogen deposition in the infected livers and spleens at 72 h of MHV-3 infection was analyzed by immunohistochemistry. * $p < 0.05$, ** $p < 0.001$. Scale bar 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

however, the FGL2 levels in the organs of TNFR2^{-/-} mice were comparable to that of WT littermates, as detected by western-blot (Fig. 4B and C). In parallel, ELISA showed that serum FGL2 levels in TNFR1^{-/-} mice were much lower than that in serum from WT or TNFR2^{-/-} mice at 72 h of infection (Fig. 4D). Therefore, lower fibrinogen deposition was observed in the infected livers and spleens of TNFR1^{-/-} mice, as confirmed by immunohistochemistry (Fig. 4E). Here, these parameters from the infected TNF- α ^{-/-} mice, which were resistant to MHV-3 infection [9], were used for controls. These combined results clearly demonstrated that the deficiency of TNFR1 signal reduces FGL2 secretion following MHV-3 infection, thus resulting in ameliorated FGL2-dependent tissue injury and mortality.

3.4. Reduced Fas-mediated cell apoptosis in MHV-3 infected TNFR1^{-/-} mice

Fas and TNFR1 actively participate in inducing hepatocyte apoptosis, which plays an important role in the processes of severe hepatitis caused by infection [12,13]. Immunohistochemistry showed that the expression of Fas and FasL in the infected hepatocytes was enhanced following MHV-3 infection, especially in

liver tissues from WT and TNFR2^{-/-} mice. However, slight expression of Fas and FasL in the infected hepatocytes was observed in TNFR1^{-/-} mice (Fig. 5A). Therefore, the level of cell apoptosis was much lower in the infected TNFR1^{-/-} livers compared to cell apoptosis in livers from TNFR2^{-/-} and WT mice at 72 h of MHV-3 infection (Fig. 5B). Similar results were observed in the infected spleen (data not shown). These combined results suggest that the expression of Fas, which mediates hepatocyte apoptosis, was also controlled by TNFR1 signal.

3.5. Inhibited neutrophil infiltration into the site of inflammation in MHV-3 infected TNFR1^{-/-} mice

Neutrophils are one type of polymorphonuclear leukocyte and are well recognized as the major player during acute inflammation [14]. Here, immunofluorescent double staining showed that Gr-1⁺ cells (indicated neutrophils) could produce several types of pro-inflammatory factors, including TNF- α , IFN- γ , IL-6 and FGL2 (Fig. 6A). To determine whether neutrophils are involved in the progression of FH following MHV-3 infection, the number of neutrophils in the infected spleens and livers of these mice were analyzed. Flow cytometry showed that high levels of neutrophils were infiltrated

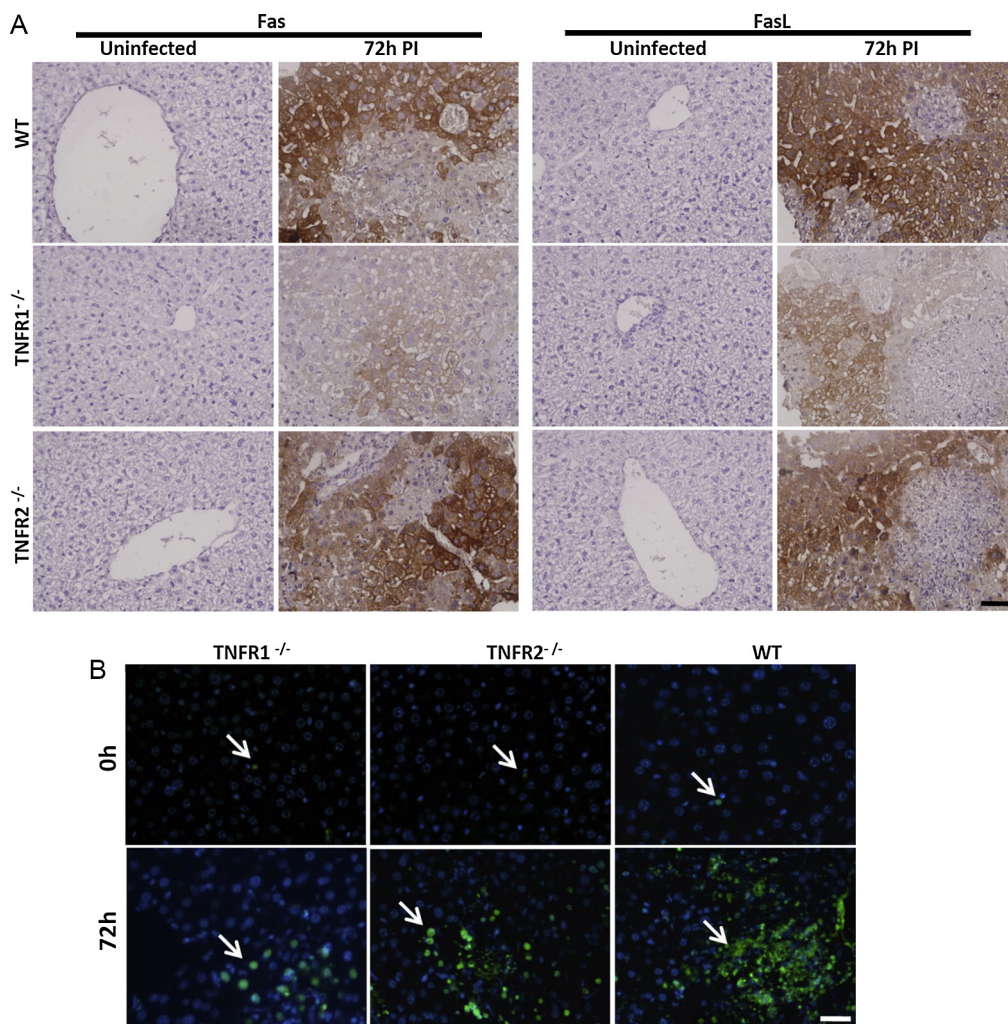


Fig. 5. Hepatocyte apoptosis was mediated by Fas/FasL signal. TNFR1^{-/-} and TNFR2^{-/-} mice as well as wild-type (WT) littermates (strain C57BL/6) were infected with MHV-3, the liver tissues were isolated at 72 h and (A) the expression of Fas and FasL was analyzed by immunohistochemistry. (B) The hepatocyte apoptosis was analyzed by using Terminal Transferase dUTP Nick End Labeling (TUNEL) staining. Scale bar = 20 μ m; arrow indicates positive cells; blue color indicates nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

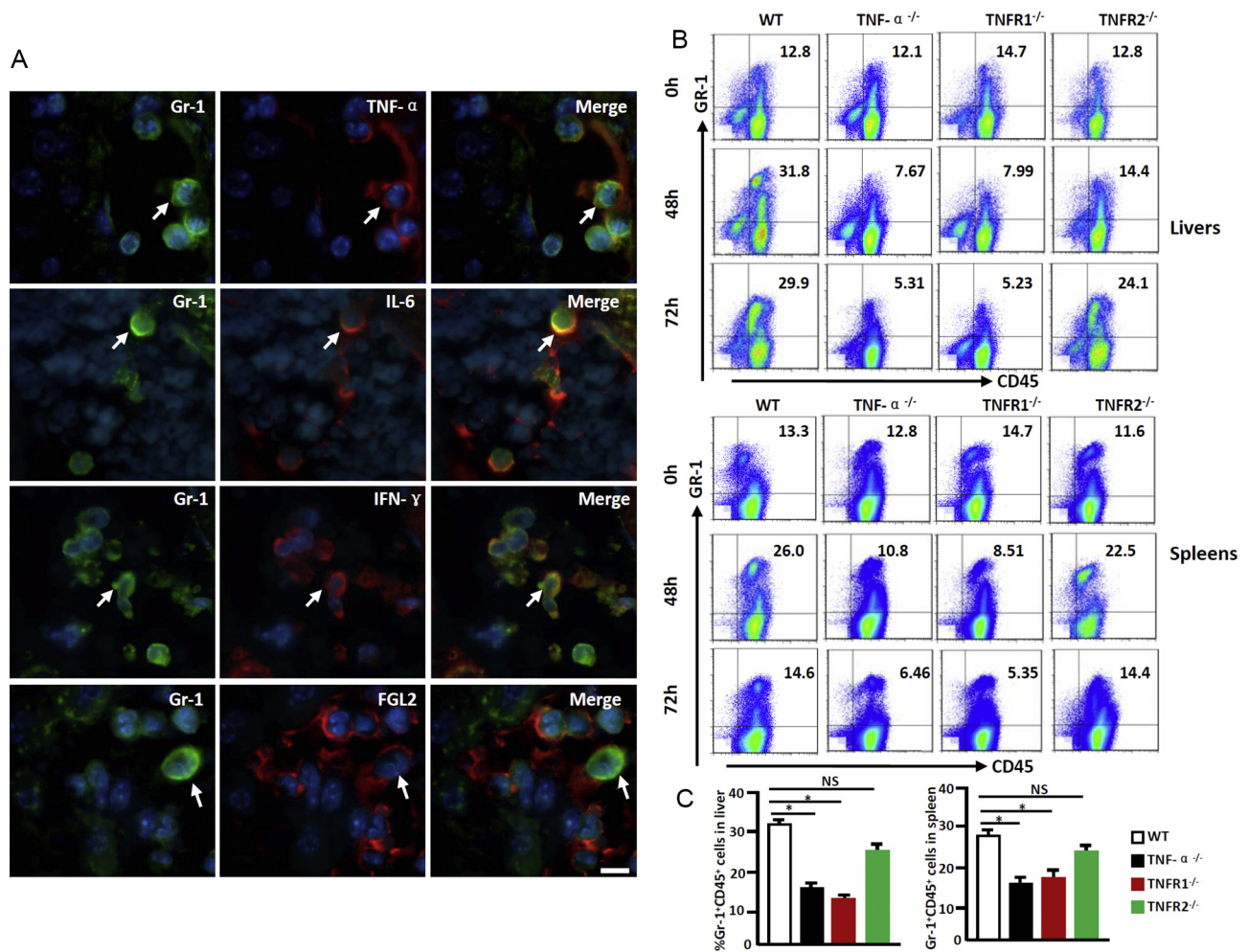


Fig. 6. The infiltration of neutrophils into the livers and spleens was mediated by TNF- α /TNFR1 signal. TNF- $\alpha^{-/-}$, TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ mice as well as wild-type (WT) littermates (strain C57BL/6) were infected with MHV-3, the liver tissues were isolated at 72 h, and (A) the co-expression of Gr-1 (the special marker for neutrophils) and inflammatory factors including TNF- α , IL-6, IFN- γ and FGL2 in the cells within WT livers was detected by immunofluorescent double staining. Scale bar = 20 μ m; arrow indicates positive cells; blue color indicates nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). (B) The number of Gr-1 $^{+}$ CD45 $^{+}$ neutrophils infiltrated into livers and spleens was analyzed by flow cytometry. Number indicated positive cells in the indicated gate. (C) The number of Gr-1 $^{+}$ CD45 $^{+}$ neutrophils infiltrated into virus-infected livers and spleens at 24 h of MHV-3 infection among these mice were counted and compared. * p < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

into livers and spleens of these mice following MHV-3 infection, especially at 72 h (Fig. 6B). However, lower numbers of neutrophils were observed in the livers and spleens of TNFR1 $^{-/-}$ and TNF- $\alpha^{-/-}$ mice, as compared to WT littermates (Fig. 6C). These results suggest that the infiltration of neutrophils into the infected organs following MHV-3 infection was controlled by TNFR1 signal.

CD4 $^{+}$ Foxp3 $^{+}$ regulatory T cells (Tregs) play an important role in the pathogenesis of FH induced by MHV-3 because they can produce FGL2 directly [15]. Moreover, adoptive transfer of Tregs from WT mice into fgl2 $^{-/-}$ mice resulted in increased mortality following MHV-3 infection [16]. To determine whether tissue infiltration of Tregs was affected by TNFR1 signaling, the number of Treg in the spleen of these infected mice was analyzed. Immunofluorescent staining showed that Tregs infiltrated into the livers and spleens of virus-infected mice (Fig. 7A). However, its levels in these organs were similar among all mouse strains (Fig. 7B). Flow cytometry also showed that high levels of Tregs were infiltrated into liver and spleen following MHV-3 infection, but their numbers among these mice are identically (Fig. 7C). These results suggest that Treg infiltration in the infected organs was not regulated by TNFR1 signals in response to MHV-3 infection.

4. Discussion

The underlying immune mechanisms that contribute to the development of FH in humans remain poorly understood because only a few cases of the disease have occurred. Thus, the successful development of a small animal model that closely resembles the key pathological findings of the human disease has significantly improved our ability to understand the pathogenesis [17]. Fortunately, a recently developed mouse FH model caused by MHV-3 infection is mainly characterized by monocyte/macrophage activation along with significantly increased production of proinflammatory mediators, and subsequent results in hepatosinusoidal thrombosis and hepatocellular necrosis. This mouse FH model exhibits a syndrome that is very similar to the clinical manifestations of FH in patients and is essential for improving our understanding of the disease pathogenesis and for designing new therapeutic strategies. In the present work, we found that MHV-3 stimulated the expression of proinflammatory factor TNF- α and its receptors, TNFR1 and TNFR2, in the infected spleens and livers, and TNF- α induced liver tissue damage and mouse mortality by stimulating FGL2 expression and fibrinogen deposition in the infected liver. Conversely, TNFR1, rather than TNFR2, deficiency

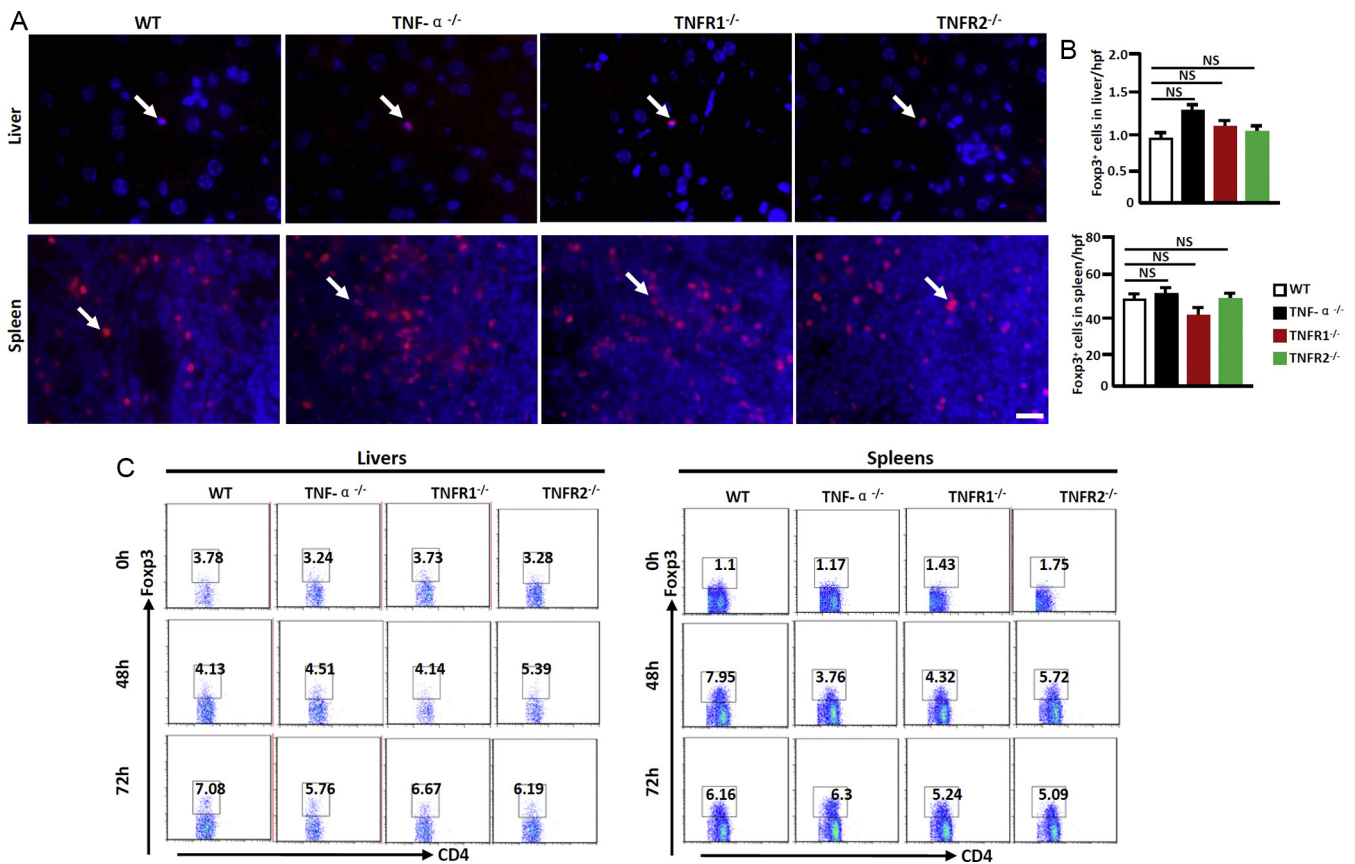


Fig. 7. The infiltration of Fopx3⁺ Tregs into the livers and spleens was not controlled by TNF- α /TNFR1 signal. TNF- α ^{-/-}, TNFR1^{-/-} and TNFR2^{-/-} mice as well as wild-type (WT) littermates (strain C57BL/6) were infected with MHV-3, the liver tissues were isolated at 72 h, and (A) the expression of Fopx3 in liver and spleen tissues was detected by immunofluorescent staining. Scale bar = 20 μ m; arrow indicates positive cells; blue color indicates nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). (B) The number of Fopx3⁺ cells that infiltrated into virus-infected livers and spleens among these mice were counted and compared. * p < 0.05, NS: not significant different. (C) The number of CD4⁺Fopx3⁺ Tregs infiltrated into liver and spleen among these mice following MHV-3 infection were analyzed by flow cytometry. Number indicated positive cells in the indicated gate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

reversed these effects, as displayed by ameliorated liver damage and reduced mouse mortality. To the best of our knowledge, the present data provided the first demonstration of TNFR1 signaling in the context of MHV-3 infection.

In inbred laboratory mice, the MHV-3 virus produces strain-dependent disease profiles that depend on the infection route, age, genetic background, and especially, the immune status of the host. Recent studies showed that FGL2 is a critical mediating factor of lethality in the MHV-3-induced FH, as demonstrated by pretreatment with neutralizing antibodies against FGL2 or interruption of its expression prevents sinusoidal fibrin deposition and hepatocellular necrosis, thereby reducing mortality in infected mice [4]. Previous work has shown that the secretion of TNF- α was initiated by MHV-3 both *in vivo* and *ex vivo*, and high concentrations of TNF- α promote the pathogenesis of FH [9]. However, TNF- α is a pleiotropic cytokine that exerts its actions *via* two distinct cell surface receptors, TNFR1 and TNFR2, which are expressed in a variety of cells. TNFR1 and TNFR2 share significant homology in the extracellular domain, while their cytoplasmic regions are quite differences. TNFR1 contains a death domain in its cytoplasmic region, but this domain is absent in TNFR2. Therefore, TNFR1 can induce cell death signals [18]. As similar to other fulminant hepatitis model [19], the expression of TNFR1 and TNFR2 in the hepatocytes was up-regulated following MHV-3 infection. To better understand which receptor transmits the TNF- α signal to promote FGL2 in this model, TNFR1^{-/-} and TNFR2^{-/-} mice were infected with MHV-3. Interestingly, a lower concentration of FGL2 was observed in the TNFR1^{-/-} liver and serum than in TNFR2^{-/-} and WT littermates,

suggesting that the secretion of FGL2 was regulated by signals from TNFR1 following MHV-3 infection. However, we here showed that not all of TNFR1^{-/-} mice resistant to MHV-3 infection and only 57% mice were survival. The morphology analysis showed that the survivors have normal liver morphology or slight liver damage, this effect was due to low FGL2 level in serum and liver tissues. Nevertheless, severe liver damage and high level of FGL2 was observed in the MHV-3 infected no-survivor of TNFR1^{-/-} mice (data not shown), suggesting other mediators from the hosts control MHV-3 virus infection and the virus mediated FGL2 secretion.

Activation of Fas and TNF affects the severity of hepatocyte apoptosis, which plays an important role in the processes of FH. Research has demonstrated that cytokines, such as TNF- α and interferon (IFN)- γ , induce hepatocyte apoptosis in MHV-3-induced FH in susceptible mice, and this induction is dependent on the induction of FGL2. This observation provides important information regarding the pathogenesis of hepatocyte injury [11,20]. In this study, the relationship between the expression of TNF receptors and apoptosis of hepatocytes was investigated in mice during the early stage of MHV-3 infection. A significant increase of hepatocyte apoptosis and high expression of TNFR1 and Fas were observed in livers at 72 h after infection. In contrast, a significant reduction in apoptotic hepatocytes was observed in TNFR1^{-/-} mice together with low levels of Fas and FasL in the tissues, suggesting that the absence of TNFR1 signal could reduce Fas-mediated hepatocyte apoptosis, in addition to impeding FGL2 secretion.

Neutrophils are the first line of immune defense against most classes of pathogenic microorganisms. Neutrophils migrate from

the bloodstream through the vascular endothelium to their target in response to inflammation [21]. At the inflammatory site, neutrophils cause necrotic tissue destruction by induced expression of a number of mediators including reactive oxygen species, complement components, and proteases as well as a variety of cytokines (e.g., TNF- α , IL-1 β and IL-12) and chemokines (including macrophage-inflammatory protein (MIP)-1 α , MIP-1 β and IL-8) [21]. The involvement of neutrophils in liver diseases, including hepatic ischemia-reperfusion injury [22], endotoxemia [23], and alcoholic hepatitis [24], has been illustrated. In this model of liver injury caused by MHV-3 infection, the number of neutrophils that infiltrate into the livers and spleen was enhanced in response to MHV-3. However, their level was much lower in tissues from TNFR1^{-/-} mice. Gr-1⁺ neutrophils contribute to the production of proinflammatory factors such as TNF- α , IFN- γ , IL-6 and FGL2. Accumulation of neutrophils in the infected organs suggest that the neutrophils contribute to liver damage, and one of the reasons that TNFR1^{-/-} mice are resistant to MHV-3-mediated FH is likely due to reduced neutrophil infiltration.

It is well established that Foxp3⁺ Tregs play a critical role in the maintenance of immunological homeostasis and in the prevention of autoimmune disorders [25]. The transcription factor Foxp3 is a unique marker specific for the Treg lineage, and deletion of Foxp3 leads to lethal multi-organ autoimmune disorders in mice and humans [26]. Recently, the expression of FGL2 has been observed from Tregs and it has been proposed to have a role in Treg effector function, as demonstrated by the fact that blocking FGL2 interrupted Treg activity *in vitro* and decreasing MHV-3-induced mortality *in vivo* [16,27]. Nevertheless, accumulating evidence shows that TNF- α preferentially up-regulates TNFR2 expression on Tregs [28], and signaling through TNFR2 promotes Treg activity and proliferation [29]. Furthermore, TNFR2 is critical for the *in vivo* immunosuppressive function of naturally occurring Tregs [30]. To determine whether the tissue infiltration and distribution of Treg was affected by the TNFR1 signal, the number of Tregs in the spleen of infected mice were analyzed, and the results showed that the infiltration of Tregs into spleens and livers was similar among all mouse strains, suggesting that Treg infiltration into the infected organs was not regulated by TNFR signals.

In conclusion, our data elucidate an important feature of TNFR1, which could act to inhibit hepatocyte apoptosis and necrosis by reducing Fas and FGL2 levels as well as impeding neutrophil and Treg infiltration during MHV-3 infection. Our study also suggests a therapeutic strategy for FH patients whereby TNFR1 signaling pathways are interrupted.

Funding

This work was supported by grants from National Natural Science Foundation of China (NSFC Nos. 81222023 and 81171585) and the U.S.-China Biomedical Collaborative Research (81361120400).

Acknowledgments

We thank Prof. Q Ning (Institute of Infectious Disease, Tongji Hospital of Tongji Medical College, Wuhan, China) kindly give us the MHV-3 virus and she also gave the research some invaluable suggestions.

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