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Original Article

Complement C5a/C5aR pathways enhance macrophage apoptosis via activation of caspase-3

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Abstract: Infection of susceptible mouse strains by murine hepatitis virus 3 (MHV-3) can cause severe acute hepatitis that reproduces the clinical syndrome of acute liver failure in patients with fulminant hepatitis (FH). Macrophages are major target cells during MHV-3 infections in mice. The complement system plays a vital role in the innate response. In this study, the function of complement C5a/C5aR pathways on MHV3-induced inflammatory response in macrophages was explored using both the murine macrophages line ANA-1 cells and C5aR-deficient mice as models. Results showed that the combination of C5a and MHV-3 significantly reduced cell viability, further confirmed to be due to increased cell apoptosis, according to the FACS assay of Annexin-V/7-AAD double staining and Western blotting. Moreover, treatment with C5a effectively increased MHV3-induced TNF-α and IL-1β secretion in ANA-1 cells. Accordingly, the C5aR deficiency caused a significant decrease in MHV3-induced TNF-α and IL-1β production in mice. Present evidence suggests that C5a/C5aR pathways can enhance MHV3-induced inflammatory cytokine production to upregulate apoptosis via caspase-3 signaling pathways, indicating that C5aR antagonist could be a potential immunoregulatory agent against MHV3-induced macrophage apoptosis.

Keywords: Complement C5a/C5aR, macrophages, inflammatory response, apoptosis

Introduction

Fulminant hepatic failure is characterized by the development of severe liver injuries with impaired synthetic capacity and encephalopathy in patients with previously normal livers, or at least well compensated liver disease [1]. Current evidence indicates the clinical syndrome of acute liver failure produced by fulminant viral hepatitis can be reproduced in mice by infection with murine hepatitis virus strain3 (MHV-3) [2]. MHV-3 is a member of the coronaviridae family, a group of positive stranded and enveloped RNA viruses. BALB/c and C57BL/6 mice are sensitive strains, with MHV-3 infection resulting in fatal acute fulminant hepatitis [3].

Macrophages are major target cells of MHV-3 infections. MHV3-induced hepatitis depends upon macrophage activation and inflammatory cytokine production [4]. On the other hand, C5a is one of the major biologically active components of the complement cascade downstream of C3, exerting its functions mainly via the canonical C5a receptor (C5aR, CD88). C5a induces chemotaxis of numerous cell types, including mast cells and macrophages [5].

Many studies have indicated that C5a engages in many diseases, like sepsis and cerebral malaria, due to the production of excess inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) [6-8]. IL-1β is an important component in the initiation and enhancement of inflammatory response, while TNF-α mediates inflammatory tissue injuries [9-11]. TNF-α and IL-1β play a significant role as signaling or effect molecules, both in the physiology and pathophysiology of autoimmune inflammatory disorders [12, 13]. Furthermore, side-effects typically associated with viraemia, including fever, rigors, headaches, and fatigue, have been observed in early trials of TNF in cancer patients [14, 15]. However,

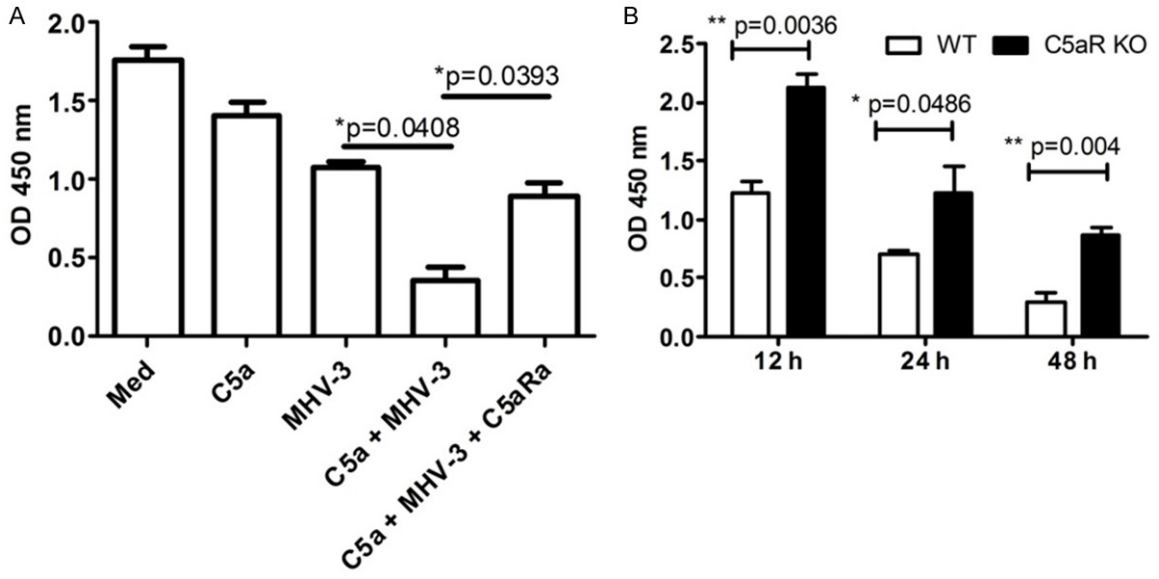

Figure 1. Effects of C5a/C5aR on MHV3-induced macrophage viability. A. After pretreatment with or without C5aR antagonist (100 nM) for 1 hour, ANA-1 cells (2×10^4/well) were seeded in flat-bottom 96-well microtiter and treated with C5a (480 ng/ml) and MHV-3 (1000 PFU) alone or in combinations for 40 hours. B. Peritoneal exudates macrophages were isolated from WT BALB/c and C5aR KO BALB/c mice and infected with 1000 PFU MHV-3 at indicated times. Cell viability was detected by MTT assays. Results are shown as mean ± SEM. *P < 0.05; **P < 0.01. All experiments were repeated three times.

how C5a/C5aR pathways correlate with inflammatory cytokines in macrophages to affect MHV3-infected macrophage survival and how they cause fulminant hepatitis remains unknown. The present study investigated the roles and underlying mechanisms of C5a/C5aR pathways in MHV3-induced macrophage survival using the murine macrophage cell line ANA-1 and C5aR-deficient mice as models.

Results

Effects of C5a/C5aR pathways on MHV3-induced macrophage viability

MHV-3 was replicated in macrophages after infections, leading to target cell death (Supplementary Figure 1A, 1B). To investigate how C5a/C5aR pathways participate in MHV3-induced macrophage viability, MTT assays were performed to determine the effects of C5a and MHV-3 alone, or in combination treatment, on ANA-1 cell growth. Results suggest that, although C5a or MHV-3 alone had some inhibition in cell viability, the combination of C5a and MHV-3 significantly suppressed cell viability (**Figure 1A**, MHV-3 & C5a + MHV-3: P = 0.0408). After pretreatment with C5aR antagonist (C5aRa) for 1 hour before cell exposure to C5a + MHV-3, cell viability was significantly increased, compared with that of C5a + MHV-3 treatment (**Figure 1**, C5a + MHV-3 & C5a + MHV-3 + C5aRa: P = 0.0393). According to the reduced cell viability after C5a treatment, increased cell viability was observed in MHV3-infected primary peritoneal exudates macrophages from C5aR-defficient mice (**Figure 1B**). Present results suggest that C5a/C5aR interaction potentiates MHV3-induced inhibition in macrophage growth.

Increased macrophage apoptosis after the combination of C5a and MHV-3 treatment

Apoptosis is known as one of the major types of cells death. To further investigate if cell apoptosis was induced by C5a and MHV-3 treatment, flow cytometry was performed by double labeling assay (FITC-Annexin-V combined with 7-AAD). As shown in **Figure 2**, compared with C5a, or MHV-3 alone, the combination of C5a and MHV-3 treatment in ANA-1 cells led to a remarkable increase in the percentage of early apoptotic cells (**Figure 2**, C5a & C5a + MHV-3: 1.12% & 13.8%; MHV-3 & C5a + MHV-3: 1.47% & 13.8%), demonstrating that reduced cell viability by the combination of C5a and MHV-3 treatment was due to increased cell apoptosis.

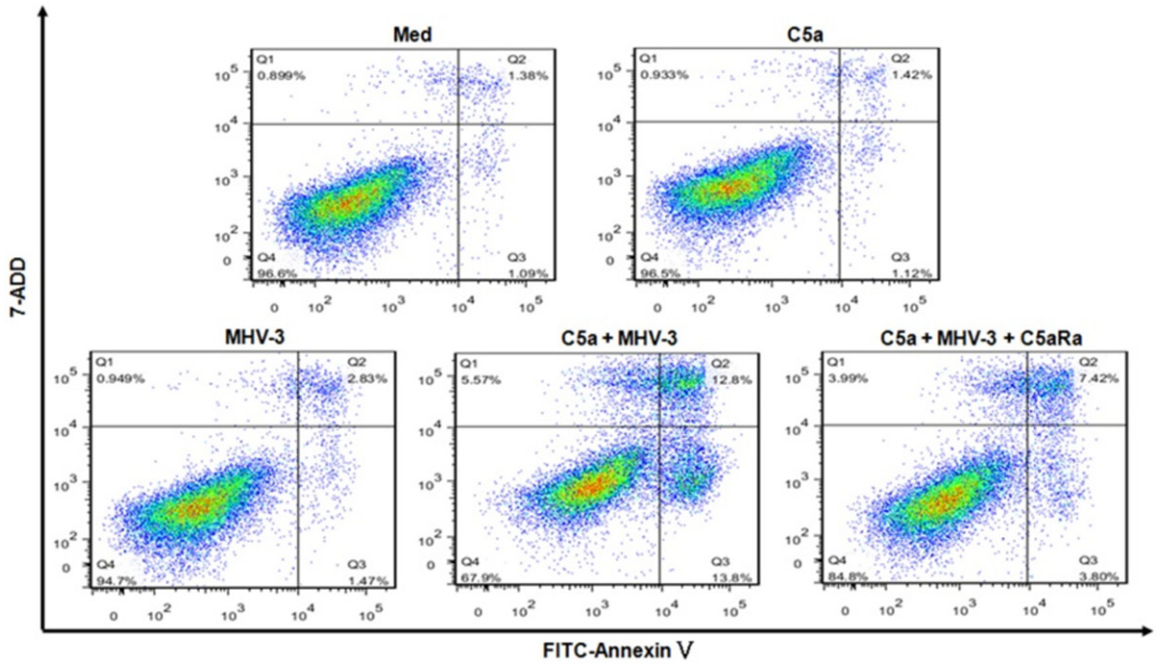

Figure 2. Effects of C5a on MHV3-induced macrophage apoptosis. After pretreatment with or without C5aR antagonist (100 nM) for 1 hour, ANA-1 cells (2×10^5/well) were seeded in flat-bottom 48-well microtiter and treated with C5a (480 ng/ml) and MHV-3 (1000 PFU) alone or in combinations for 40 hours. Cells were collected and stained by Annexin-V and 7-AAD. The apoptotic percentage was analyzed by flow cytometry. Data are representative of two independent experiments.

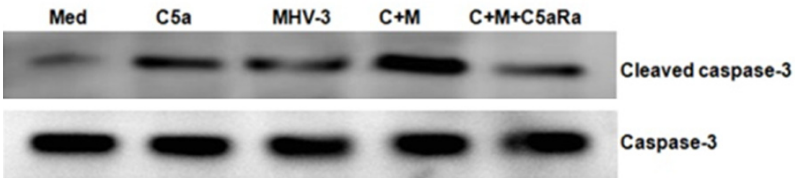

Figure 3. Caspase-3 activation is involved in C5a and MHV3-mediated macrophage apoptosis. ANA-1 cells were treated with C5a, MHV-3, C5a + MHV-3, or C5a + MHV-3 + C5aRa (Cells were pretreated with C5aRa for 1 hour) for 40 hours. Expression of Caspase-3 and cleaved Caspase-3 was measured by Western blot. (C5a: 480 ng/mL; MHV-3: 1000 PFU; C5aRa: 100 nM).

Increased caspase-3 in macrophage activation after the combination of C5a and MHV-3 treatment

Previous studies have shown that caspase-3 plays a critical role in the execution of apoptosis [16]. To investigate the relationship of caspase-3 with C5a and MHV3-induced macrophage apoptosis, caspase-3 activation was probed with Western blotting. As shown in **Figure 3**, the cleaved caspase-3 subunit was obviously increased in C5a and MHV3 combination-treated ANA-1 cells while the addition of C5aR antagonist reversed these potentiating effects.

Involvement of C5a/C5aR pathways in MHV3-mediated inflammatory cytokines production by macrophages

C5a/C5aR axis regulates expression of TNF-α and IL-1 [17, 18]. TNF-α is a critical inflammatory cytokine for MHV3-induced fulminant hepatitis [19]. Consistent with previous studies, it was found that TNF-α deficiency led to a remarkable increase in survival in MHV3-infected mice (**Figure 4**). The production of TNF-α (**Figure 5A**, MHV-3 & C5a + MHV-3: P = 0.0302; C5a + MHV-3 & C5a + MHV-3 + C5aRa: P = 0.0016) and IL-1β (**Figure 5A**, MHV-3 & C5a + MHV-3: P = 0.0104; C5a + MHV-3 & C5a + MHV-3 + C5aRa: P = 0.0011) by the combination of C5a and MHV3- treated ANA-1 cells was significantly higher than that of C5a, or MHV-3 alone-treated ANA-1 cells. Accordingly, C5aR-deffiiciency caused significantly lower TNF-α (**Figure 5B**, WT & C5aR KO: P = 0.0002) and IL-1β (**Figure 5B**, WT & C5aR KO: P = 0.0008) secretion in mice after MHV-3 infection. The combi-

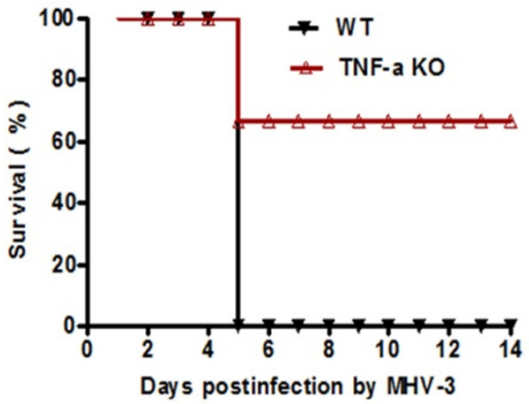

Figure 4. TNF-α deficiency attenuates mice susceptibility to MHV-3 infections. Wild type (WT) C57BL/6 and TNF-α knockout (TNF-α KO) mice were infected with 100 PFU of MHV-3. The survival rate was monitored for a total of 14 days (n = 5/group). Representative data from two independent experiments are shown.

nation of TNF-α and IL-1β, or TNF-α and IL-1β alone, *in vitro*, led to a marked inhibition in ANA-1 cell viability (**Figure 6**, Med & TNF-α: P = 0.0059; Med & IL-1β: P = 0.0115; Med & TNF-α + IL-1β: P = 0.0047). Present results indicate that C5a/C5aR pathways are involved in MHV3-induced inflammatory cytokine response.

Discussion

Recent studies have demonstrated that C5a, a main complement component, affects various diseases [20-22], including MHV3-induced FH [19]. A previous study also suggested that the cellular signaling and responses in C5a-targeted macrophages, triggered by C5aR activation, are critical for MHV3-induced FH [23]. However, how the complement C5a/C5aR pathways regulate MHV3-infected macrophage function remains unclear. In the present study, using macrophage cell line ANA-1 cells and C5aR-defficient mice as models, results clearly demonstrate that C5a/C5aR pathways can enhance MHV3-induced macrophage apoptosis via caspase-3 signaling pathways.

C5a executes its biological actions by binding to its specific receptor C5aR (CD88), which is expressed on a variety of inflammatory cells [24], including macrophages. C5aR binds to the complement activation product C5a and mediates pro-inflammatory actions [25]. In a murine model of ischemia/reperfusion (I/R) injury, inhibition of C5aR has been found to diminish the *in vivo* production of TNF-α [26]. Consistent with these results, the current study found that inflammatory cytokines TNF-α and IL-1β production was significantly reduced in MHV3-infected $C5aR^{-/-}$ mice, while recombinant C5a enhanced MHV3-induced TNF-α and IL-1β secretion by ANA-1 cells, demonstrating a critical role for C5a/C5aR in MHV3-induced TNF-α and IL-1β production.

When combined with IFN-γ or IL-1β, TNF-α induces a variety of cell apoptosis, including β-cell and gingival fibroblasts [27, 28]. It was found that C5a treatment enhanced IL-1β and TNF-α production and increased cell apoptosis in MHV3-infected ANA-1 cells. Caspase-3 is the most extensively studied apoptotic protein, a key effector in the apoptosis pathway. The current study found that C5a treatment led to an obvious increase in caspase-3 activation in MHV3-infected ANA-1 cells, suggesting that C5a/C5aR pathways potentiate MHV3-induced ANA-1 cell apoptosis via caspase-3 activation.

Hexapeptide AcF(OP[D]ChaWR is a C5aR antagonist that has been shown to be effective in the treatment of autoimmune diseases in preclinical research [29, 30]. Current results showed that C5aR antagonists significantly improved ANA-1 cell viability and reduced inflammatory cytokine production and cell apoptosis. Present results demonstrate that interfering with C5aR signaling may be a potential strategy for prevention of MHV3-induced macrophage apoptosis.

Materials and methods

Plaque reduction neutralizing test

The neutralizing ability of MHV3-infected cells was measured with a plaque reduction neutralizing test (PRNT). Briefly, ANA-1 cells (1×10^6 cells/well) were seeded in 6-well microtiter plates and infected with 1000 PFU MHV-3. Virus-serum mixture was harvested at indicated time points. 17Cl-1 cells with 90% confluence in 24-well plates were infected with the tenfold serial dilutions of the above virus-serum mixture (1:1-1:1000) at 37°C for 72 hours. Plaques were then counted after staining with crystal violet.

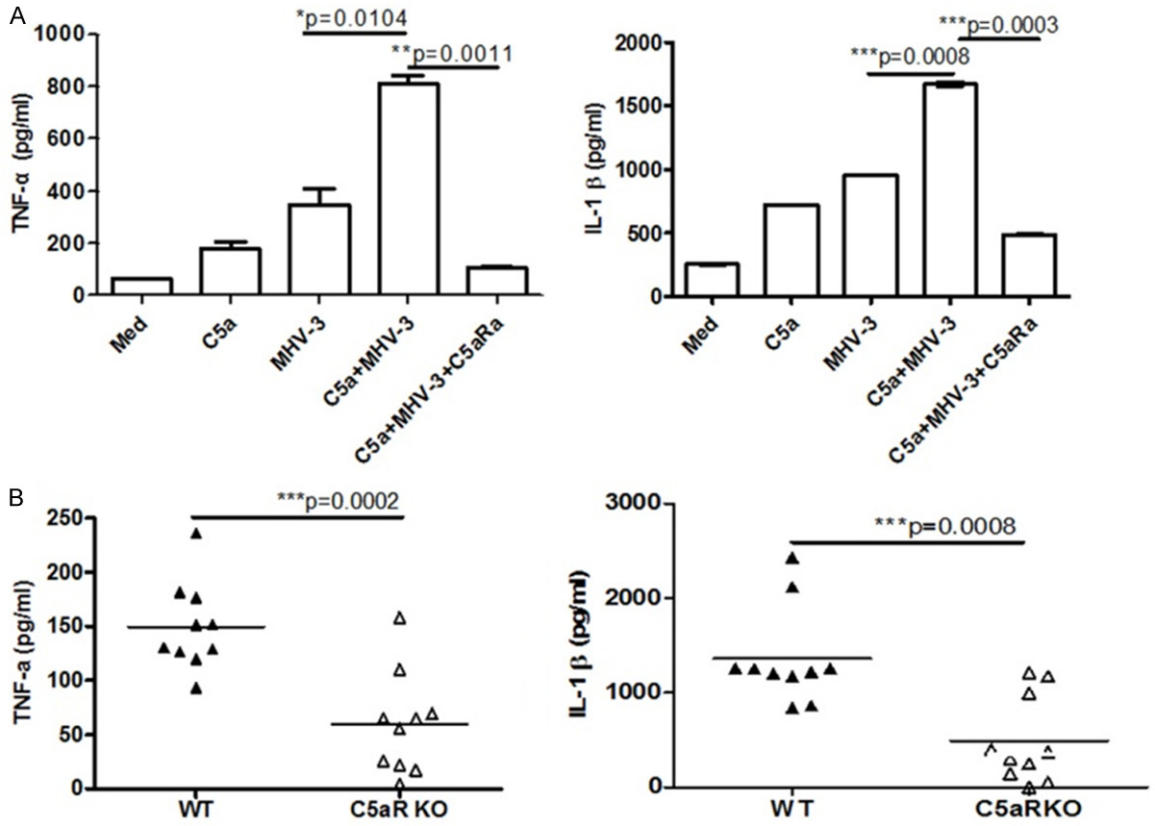

Figure 5. Effects of C5a/C5aR pathways on MHV3-induced TNF-α and IL-1β production. (A) Effects of C5a on TNF-α and IL-1β production in MHV-3-induced ANA-1 cells. Cells were treated with C5a, MHV-3, C5a + MHV-3, or C5a + MHV-3 + C5aRa (Cells were pretreated with C5aRa for 1 hour) for 40 hours (n = 3/group, C5a: 480 ng/ml; MHV-3: 1000 PFU; C5aRa: 100 nM). (B) C5aR-defficency led to a reduced TNF-α and IL-1β production in MHV3-infected mice. Wild type (WT) BALB/c and C5aR knockout (C5aR KO) mice (n = 10/group) were infected with 100 PFU of MHV-3 for 72 hours. TNF-α (A) and IL-1β (B) levels in the culture supernatant and serum were measured by enzyme linked immuno-sorbent assay (ELISA). Results are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.0001. Representative data from two independent experiments are shown.

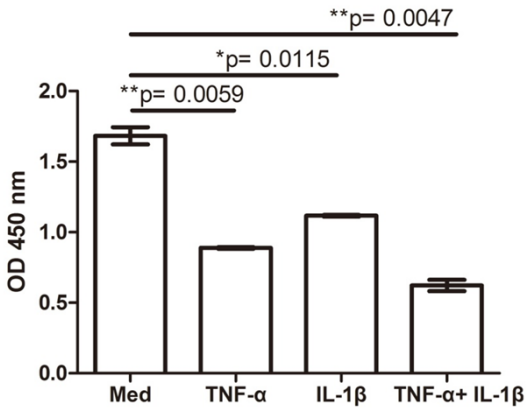

Figure 6. Effects of TNF-α and IL-1β on macrophage viability. ANA-1 cells (2×10^4/well) were seeded in flat-bottom 96-well microtiter and treated with TNF-α (10 ng/ml) and IL-1β (17.5 ng/ml) alone or in combinations for 40 hours. Cell viability was assessed by MTT assays. Results are shown as mean ± SEM. *P < 0.05; **P < 0.01. Representative data from two independent experiments are shown.

Cell culture

The ANA-1 murine macrophage cell line (origin of this macrophage cell line was from MHV-3 relatively susceptible C57BL/6 mice) was obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were grown in RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum (Hyclone, USA), 100 units/mL penicillin, and 100 ug/mL streptomycin. They were maintained at 37°C in 5% CO_2.

Morphological assessment of MHV3-infected ANA-1 Cells

Cells (2×10^5 cells/well) were seeded in 24-well microtiter plates and infected with 1000 PFU MHV-3. Morphological changes of live cells were observed under phase contrast micro-

scope (Olympus 1X71S8F-2, Japan) at indicated time points.

Assessment of cell viability

ANA-1 cells were seeded in a 96-well plate with 2×10^4 cells/well and treated with 480 ng/ml C5a (Biovision, USA) or 1000 PFU MHV-3 (kindly provided by Prof. Ning, Institute of Infectious Disease, Tongji Hospital of Tongji Medical College, Wuhan, China). They were treated alone or in combination or pretreated with 100 nM C5aR antagonist (GL Biochem, China) for 30 minutes before C5a and MHV-3 in combination for 40 hours. In some experiments, cells were treated with 10 ng/mL of TNF-α or 17.5 ng/mL of IL-1β (Peprotech, CA, USA) alone, or in combination for 40 hours. Cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays.

To measure MHV3-induced primary macrophage viability from resistant and susceptible animals, this study isolated peritoneal exudates macrophages from WT BALB/c and C5aR KO BALB/c mice. After MHV-3 infection at indicated times, cell viability was detected by MTT assay.

Assessment of TNF-α and IL-1β secretion

ANA-1 cells were seeded at 1×10^6 cells/well in 6-well plates and treated with C5a and MHV-3, as described above, for 40 hours. Levels of TNF-α and IL-1β in culture supernatant were determined by the enzyme-linked immunosorbent (ELISA) kit (Biolegend), according to manufacturer instructions.

Analysis of cell apoptosis by flow cytometry

ANA-1 cells were seeded in a 24-well plate with 2×10^5 cells/well. They were harvested after treatment with C5a and MHV-3, as described above, for 40 hours. According to manufacturer suggested protocols, cells were double stained with Annexin-V-FITC and 7-AAD (eBioscience, CA, USA). Cell apoptosis was analyzed by flow cytometry (BD, FACS Canto II).

Western blot

ANA-1 cells were seeded in a 6-well culture plate with a 1×10^6 cells/well. They were treated with C5a and MHV-3, as described above, for 24 hours. Cells were lysed with protein lysis buffer containing a protease inhibitor. The extracted protein was electrophoresed on SDS-polyacrylamide gels (4%-12%) and transferred to nitrocellulose membranes. Membranes were incubated with anti-cleaved caspase-3 or total caspase-3 antibody (Cell Signaling) at 4°C overnight. Next, the membranes were incubated with a peroxidase-conjugated secondary antibody and visualized by a super-enhanced chemiluminescence detection system.

Mice experiments

Wild-type (WT) C57BL/6 and BALB/c mice were obtained from the Animal Institute of Academy of Medical Science (Beijing, China). TNF-α knockout (TNF-α KO) (C57BL/6 background) and C5aR knockout (C5aR KO, BALB/c background) mice were purchased from Jackson Laboratory. Eight 12-week old specific pathogen-free female mice were used for all experiments. Mice experiments were performed according to the Health Guide for Institutional Animal Care and Use Committee of Third Military Medical University. Mice were infected intraperitoneally with 100 PFU MHV-3 and sacrificed at indicated times. Serum TNF-α and IL-1β levels in MHV3-infected mice were measured by ELISA.

Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. Data are expressed as the mean ± SEM. Significant differences between control and experimental groups were analyzed by t-test. Differences with a p value less than 0.05 are considered statistically significant.

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Disclosure of conflict of interest

None.

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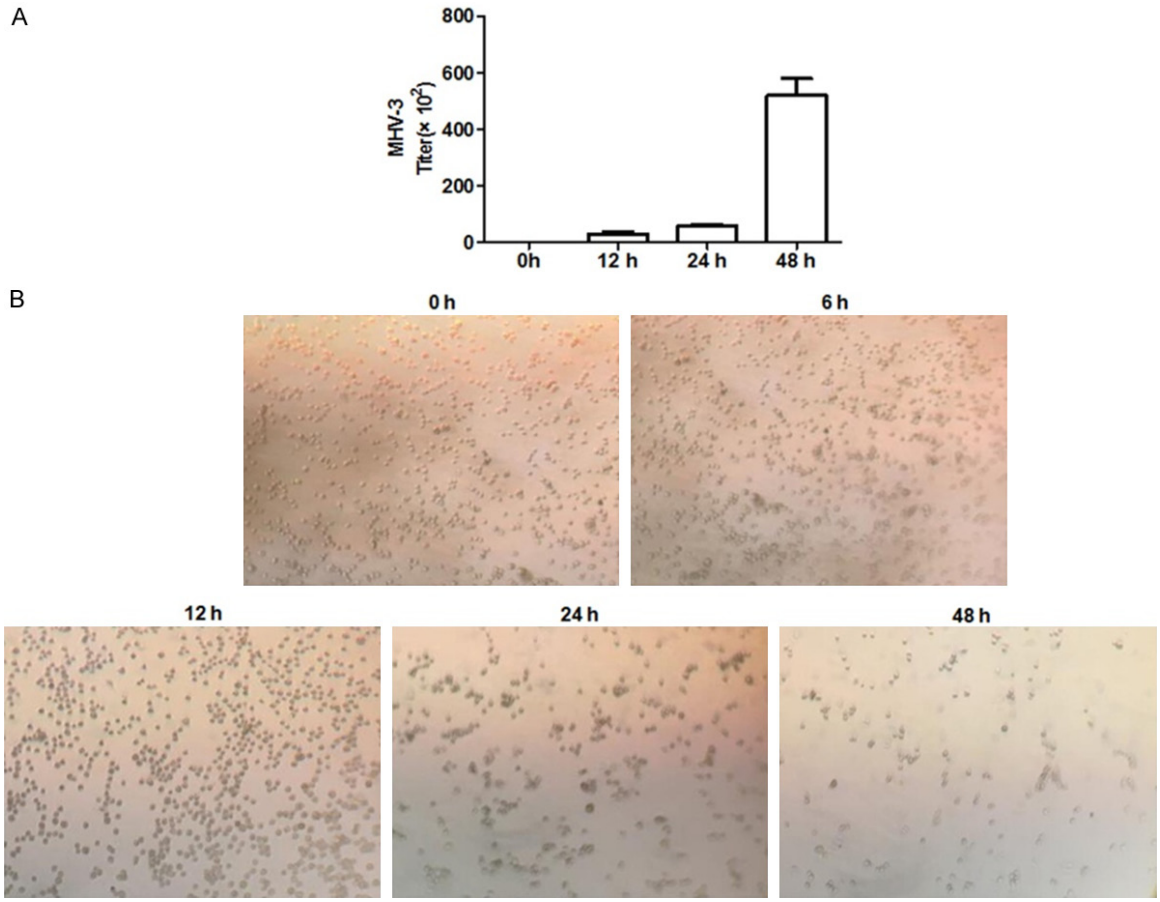

Supplementary Figure 1. A. Kinetics of virus growth in MHV3-infected ANA-1 cells. ANA-1 cells were infected with 1000 PFU MHV-3 at indicated times and the virus titration in the culture supernatant was measured by plaque reduction neutralizing test. B. MHV-3 infection led to reduced ANA-1 cell viability. ANA-1 cells were infected with 1000 PFU MHV-3 at indicated times and the morphological changes of live cells were observed under phase contrast microscope. Magnification: ×200.