Short-lived AIM2 Inflammasome Activation Relates to Chronic MCMV Infection in BALB/c Mice*

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Summary: Absent in melanoma 2 (AIM2) inflammasome is a crucial link bridging the innate host defense and the subsequent adaptive immunity when activated by exogenous double stranded DNA (dsDNA). Through establishing models of disseminated murine cytomegalovirus (MCMV) infection in BALB/c and C57BL/6 mice, we evaluated dynamic expression of AIM2 inflammasome components and its relationship with pathological damage and viral replication, trying to figure out whether AIM2 inflammasome is related to the chronic mechanism of MCMV. BALB/c and C57BL/6 mice were sacrificed on day 0, 1, 3, 7, 14 and 28 post infection. Expression levels of AIM2, pro-caspase-1, caspase-1 p20, pro-IL1β and mature IL1β in primary peritoneal macrophages (PMs) and spleens were detected by Western blotting. Contents of IL18 in the serum were detected by ELISA. Pathological examinations of livers were performed, and mRNA levels of MCMV glycoprotein B (gB) in salivary glands also assessed. Results showed that expression levels of AIM2 in PMs and spleens of C57BL/6 mice increased on day 3, even continued to day 28; caspase-1 p20 and mature IL1β increased on day 7, 14 and 28; the persistently high expression of IL18 in the serum started on day 1, showing a double peak curve. As for BALB/c mice, expression of AIM2 in PMs increased on day 1 and day 7, while contents of AIM2 in spleens increased on day 1 and day 3; caspase-1 p20 and mature IL1β merely increased 7 days fter infection. Thereafter, expression levels of AIM2, caspase-1 p20, mature IL1β and IL18 were limited; the duration of AIM2 inflammasome activation in BALB/c mice was much shorter than that in C57BL/6 mice. The severer pathological damage and more viral replications in BALB/c mice further proved the deficient antiviral immunity to MCMV. In conclusion, the activation of AIM2 inflammasome in BALB/c mice was short-lived, which is quite possibly related to the chronicity of MCMV infection.
Key words: AIM2; murine cytomegalovirus; BALB/c mice; C57BL/6 mice; macrophages

Human cytomegalovirus (HCMV) is widespread in the world. Although HCMV infection is usually asymptomatic in immunocompetent individuals, it causes serious consequences in immunocompromised individuals including transplant recipients and infants suffering intrauterine infection[1–3]. Because of the rigorous species specificity of HCMV, it is impossible to build animal models with HCMV infection for now. Fortunately, murine cytomegalovirus (MCMV) infection shares much in common with HCMV in genomes and pathogenesis, and mice infected with MCMV are widely used to study the mechanism of HCMV infection[4].

As a member of the HIN200 protein family, AIM2 comprises two functional domains: an N-terminal pyrin domain and a C-terminal HIN domain, the latter can sense exogenous dsDNA[5–7]. In most cases, AIM2 is expressed in relatively low levels in innate immune cells. Once activated, AIM2 recruits apoptosis-associated speck-like protein (ASC) containing a caspase recruitment domain to cleave pro-caspase-1 and finally converts pro-IL1β and pro-IL18 into their mature molecules, IL1β and IL18[5, 8]. As important mediators of the inflammatory response, IL1β can take effects on cellular adaptive immunity, while IL18 is essential for the induction of IFNγ[9–11]. Through the production of the two important proinflammatory cytokines, AIM2 inflammasome is crucial for innate and subsequent adaptive immune response during various virus infections including MCMV[12, 13].

MCMV infection in BALB/c mice is serious and usually ends up with chronicity, while C57BL/6 mice are resistant to MCMV and capable of removing virus in

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acute infection period; the different infection outcomes were usually attributed to specific Ly49H-m157 pathway of NK cells in C57BL/6 mice[14, 15]. Considering the huge genome of MCMV and complicated antiviral responses *in vivo*, other factors were probably involved as well. Our previous study has found different antiviral responses of NK cells and $CD8^+$ T cells in the two mouse strains after MCMV infection[16, 17]. As mentioned, AIM2 inflammasome initiated multiple immune responses. We were curious to know whether AIM2 inflammasome influenced the chronic mechanism of MCMV infection. For further confirmation, we built models of MCMV infection in BALB/c and C57BL/6 mice, which were natural chronic and acute infection models for comparing different antiviral responses. Through assessing their dynamic expression levels of AIM2 inflammasome components and changes of pathological damage and viral replication, we aimed to illuminate the relationship between AIM2 inflammasome and the chronic mechanism of MCMV infection.

1 MATERIALS AND METHODS

1.1 Animals

Female 5-week-old BALB/c and C57BL/6 mice were purchased from the local experimental animal center (Wuhan, China). Mice were housed under specific pathogen-free conditions with free access to food and water. The mice were divided into control group and infection group randomly (n=4 to 5 mice in each group). Mice of infection groups received intraperitoneal injection of 5×10^4 plaque forming unit (PFU) MCMV Smith strain and were sacrificed on day 1, 3, 7, 14 and 28 post infection. Mice of the control group received the same volume of dulbecco's modified eagle medium (DMEM). Our previous research found that expression levels of AIM2 inflammasome components in the control group were stable at different time point[18]. Therefore, mice that received DMEM 7 days before were used as the control to evaluate the activation of AIM2 inflammasome in protein extracts and serum. The pathological examination of livers was also conducted. In this case, mice of infection groups were compared with the corresponding mice, which received DMEM instead of MCMV Smith and were sacrificed after 1, 3, 7, 14 and 28 days (n=3 mice in each group). All the animal procedures were performed according to the Helsinki Declaration and Institutional Animal Care and Use Committee of Tongji Hospital.

1.2 Main Reagents

Thioglycollate Medium was obtained from Hopebio (China). AIM2 specific antibody for Western blot was obtained from Cell Signaling Technology (America). Antibodies to caspase-1 and IL1β were purchased from BioVision (America). ELISA kit of IL-18 was obtained from eBioscience (America). Trizol (Invitrogen, America), PrimeScript™ RT Master Mix and SYBR Premix Ex Taq were purchased from Takara (Japan), primers were from TSINGKE (China).

1.3 Virus and Cell Culture

MCMV Smith strain was achieved after passage in salivary glands of BALB/c mice, and the viral titer was measured by standard plaque assay[19]. Briefly, mouse embryo fibroblasts were harvested from embryos on day 14 of gestation and cultured in DMEM containing 10% fetal bovine serum (FBS). Salivary glands were homogenized and resuspended with DMEM. The supernatant of homogenized salivary glands was subjected to serial dilution and seeded in 24-well culture plates with mouse embryo fibroblasts. Plaques were calculated a few days later. Peritoneal exudate cells were prepared as reported[20, 21]. Mice received intraperitoneal injection of 2 mL of 3% thioglycolate medium 3 days before. Peritoneal exudate cells were collected by peritoneal lavage. Then cells were washed with PBS and cultured in RPMI 1640 medium supplemented with 10% FBS. After incubation in the 37°C cell incubator for 4 h, nonadherent cells were removed by washing twice with PBS. Through morphologic examination and nonspecific esterase staining, the remaining cells were considered as primary peritoneal macrophages (PMs).

1.4 Western Blotting

Contents of AIM2, pro-caspase-1, caspase-1 p20, pro-IL1β and mature IL1β (IL1β p17) in primary PMs and spleens were detected by Western blotting. Pre-experiment found that contents of IL18 in protein extracts were too low to be detected by Western blotting.

Primary PMs and spleens were lysed with RIPA buffer supplemented with protease inhibitor cocktail for 30 min. The supernatant of cell lysate was obtained by centrifuging at 12 000 rpm for 10 min at 4°C. Protein samples were subjected to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and incubated with specific primary antibodies overnight at 4°C. Then the membranes were incubated with HRP-conjugated secondary antibody. The protein signals were detected with the enhanced chemiluminescence (ECL) assay kit.

1.5 ELISA

Levels of IL18 in serum of BALB/c and C57BL/6 mice were assessed by the ELISA kit according to manufacturer's instructions (eBioscience, USA).

1.6 Pathological Examination

Livers of BALB/c and C57BL/6 mice were sectioned and fixed for at least 24 h. Then tissue samples were paraffin-embedded and sectioned into 5 μm thick slices before hematoxylin-eosin (HE)

staining. Olympus BX41 microscope was used to assess the pathological damage of livers according to the instruction of Knodell histological activity index (HAI).

1.7 Quantity Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Salivary glands were separated from BALB/c and C57BL/6 mice (n=3–5 mice in each group). Total cellular RNA was isolated with the Trizol method. cDNA was synthesized with PrimeScript™ RT Master Mix and subjected to SYBR Premix Ex Taq according to the instruction of qRT-PCR (Takara, Japan). The mRNA levels of MCMV gB were determined in this way. Primer sequences used were as follows: HPRT1, sense, GGGCTTACCTCACTGCTTTC, and antisense, TCTCCACCAATAACTTTTATGTCC; MCMV gB, sense, GCGACATACACTTCTCCATT, and antisense, CAGAATACGTGGCTCACA. PCR conditions were set as initial denature at 95°C for 30 s, followed by each cycle of melting at 95°C for 5 s, primer-annealing at 58°C for 30 s, and elongating at 72°C for 30 s. Data were normalized to HPRT1 and calculated by the method of $2^{-\Delta\Delta CT}$.

1.8 Statistical Analysis

Data were shown as mean±standard error of mean and analyzed with SPSS Statistics software version 17.0 (SPSS Inc., USA). Dunnett correction for t-test was used in comparisons between infection groups and the control group. Comparisons of the corresponding group between BALB/c and C57BL/6 mice were performed with Two-way ANOVE. P values of less than 0.05 were considered significant difference.

2 RESULTS

2.1 Different Activation Status of AIM2 Inflammasome in BALB/c and C57BL/6 Mice after MCMV Infection

We first detected the activation of AIM2 inflammasome in PMs of BALB/c mice (fig. 1A). More AIM2 was expressed in BALB/c mice on day 1 and day 7 post infection, and AIM2 expression had no difference between BALB/c group and control group on day 3, 14 and 28. Pro-caspase-1 and pro-IL1β were constitutively expressed in BALB/c mice, while caspase-1 p20 and mature IL1β increased on day

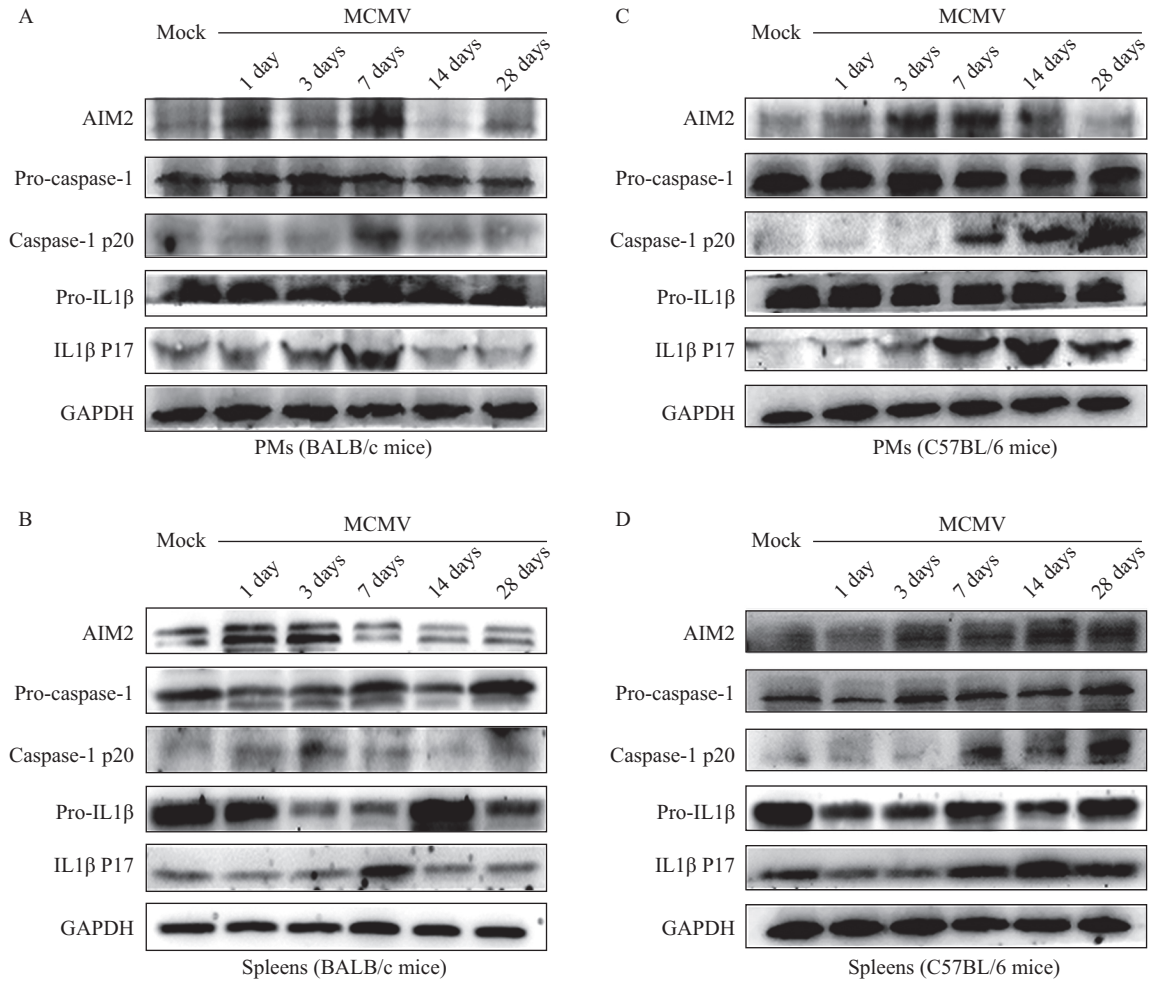

Fig. 1 The activation of AIM2 inflammasome in BALB/c and C57BL/6 mice after MCMV infection
PMs and spleens of BALB/c (A, B) and C57BL/6 (C, D) mice were harvested and the cell lysate was obtained to detect the expression of AIM2, pro-caspase-1, caspase-1 p20, pro-IL1β and mature IL1β by Western blotting.

7 post infection. Levels of caspase-1 p20 and mature IL1β in other infection groups were similar to those in the control group. We further detected the activation of AIM2 inflammasome in spleens (fig. 1B). Contents of AIM2 in BALB/c mice increased on day 1 and day 3 post infection. The expression of AIM2 reduced later, showing no difference as compared with the control group. Unlike PMs, expression levels of pro-caspase-1 and pro-IL1β were different in MCMV infection mice. Levels of caspase-1 p20 increased on day 1 and day 3 post infection. The mature form of IL1β (IL1β p17) increased on day 7.

As for C57BL/6 mice, contents of AIM2 in PMs increased on day 3, 7 and 14 post infection. The expression levels of pro-caspase-1 and pro-IL1β in C57BL/6 mice were similar to those in BALB/c mice. Notably, the increased caspase-1 p20 and mature IL1β of C57BL/6 mice appeared on day 7 and continued to day 28 post infection, which was obviously different from those in BALB/c mice (fig. 1C). In spleens, C57BL/6 mice showed an elevated level of AIM2 on day 3 and held this status until the day 28 post infection. Similarly, contents of pro-caspase-1 and pro-IL1β varied at different stages of infection. This could be the influence of many different cell types in spleens. Except for macrophages, other cells could express pro-caspase-1 and pro-IL1β. The increased expression of caspase-1 p20 and IL1β p17 started on day 7 and continued to day 28 post infection (fig. 1D).

IL18 is another crucial inflammatory factor in antiviral immune responses[11]. For some reason, we could not detect IL18 in cell lysates. It was very likely that IL18 had been secreted into the culture supernatant or blood. Therefore, we evaluated contents of IL18 in peripheral blood serum of BALB/c and C57BL/6 mice by ELISA. In BALB/c mice, IL18 only increased on day 3 post infection and held a relatively stable content in all other groups (fig. 2A). In sharp contrast, expression levels of IL18 in C57BL/6 mice were enhanced during the whole process of infection, which started on day 1 and showed a double peak curve (fig. 2B).

Compared with two mouse strains, the activation of AIM2 inflammasome in BALB/c mice lasted no longer than 7 days post infection, while C57BL/6 mice held

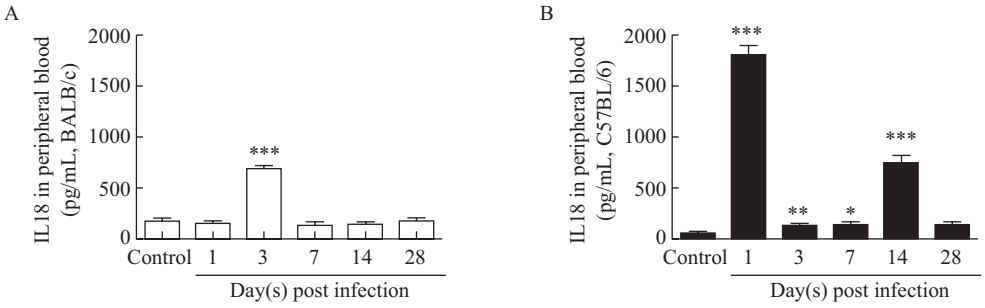

Fig. 2 The expression levels of IL18 in serum of BALB/c and C57BL/6 mice
Peripheral blood (n=3–5 each) was collected and contents of IL18 in BALB/c (A) and C57BL/6 (B) mice were detected by ELISA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* the control group

a longer duration of AIM2 inflammasome activation, which could continue to the day 28 post infection. Clearly, expression levels of AIM2 inflammasome components in the two mouse strains were different, which proved the diversity of AIM2 inflammasome activation.

2.2 Histological Analysis of Livers Showed More Serious Pathological Damage in BALB/c Mice

As a common site of MCMV infection, the liver adopts various immune strategies to resist infection[22, 23]. Pathological examination was conducted to evaluate the dissemination of MCMV in livers according to HAI (fig. 3). Pathological damage of livers in BALB/c mice was almost undetected on day 1 post infection. Then the condition worsened on day 3, 7 and 14 with obvious spotty necrosis and infiltration of inflammatory cells in portal tracts. Viral intranuclear inclusions were also visible at this stage. The pathological condition of livers on day 28 alleviated, although infiltration of inflammatory cells still existed. Livers of C57BL/6 mice were basically normal on day 1 post infection. The pathological damage became worst on day 3 with extensive hepatocyte necrosis and infiltration of inflammatory cells, then it alleviated. Till the day 28, livers of C57BL/6 mice had largely recovered. Compared with BALB/c mice, though livers of C57BL/6 mice showed more serious pathological damage on day 3 post infection, the overall pathological damage was relatively mild during MCMV infection. Histological changes of livers are shown in table 1.

2.3 BALB/c Mice Suffered More Viral Loads Than C57BL/6 Mice in Salivary Glands

The salivary gland is a common site for MCMV latency and its viral replication provides a good indicator to evaluate overall levels of viral loads in mice[24]. When infected with MCMV, BALB/c and C57BL/6 mice had similar expression trends of MCMV gB. Both of them kept increased levels of MCMV gB before the day 14

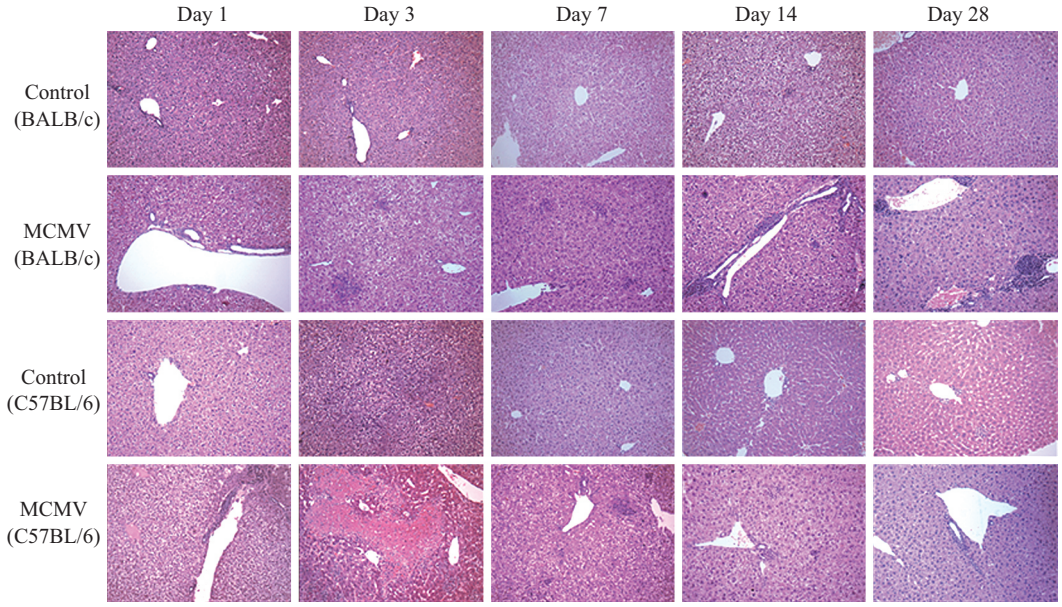

Fig. 3 Pathological changes of livers in BALB/c and C57BL/6 mice
Tissue samples were sectioned and underwent HE staining. Comparisons of the liver in the two mouse strains were assessed by HAI. Representative images of each group were shown (HE staining, 200×).

Table 1 Histological analysis of livers after infection according to HAI (*n*=3–5)

Group	HAI				
	Day 1	Day 3	Day 7	Day 14	Day 28
BALB/c	0.63±0.38	6.06±0.45	7.75±0.16	7.38±0.32	4.17±0.57
C57BL/6	0.25±0.25	8.00±0.13**	5.23±0.21**	3.46±0.47***	1.90±0.36*

*P<0.05, **P<0.01, ***P<0.001 *vs.* BALB/c mice

post infection and MCMV gB decreased later on day 28. Compared with C57BL/6 mice, BALB/c mice had a much higher viral load during the whole infection (fig. 4). Clearly, consistent with the shorter duration of AIM2 inflammasome activation in PMs and spleens, BALB/c mice suffered more serious viral infection than C57BL/6 mice in MCMV infection.

3 DISCUSSION

Macrophages are important innate immune cells which can express AIM2 and form AIM2 inflammasome after being activated by exogenous dsDNA[7, 8, 25]. The abdominal cavity of mice is a good site for collecting purer PMs and serves as a preferred sample source. The spleen is an important immune organ to control MCMV infection and expresses AIM2 constitutively, so we also detected the activation of AIM2 inflammasome in spleens to closely reflect the *in vivo* situation[12]. In BALB/c mice, the expression of AIM2 in PMs only increased at the early stage of infection (day 1 and day 7 post infection). Contents of AIM2 in spleens increased on day 1 and day 3. Caspase-1 p20 and mature IL1β had a higher level on day 7 post infection. IL18 in serum of BALB/c mice merely increased on day 3 and maintained persistently

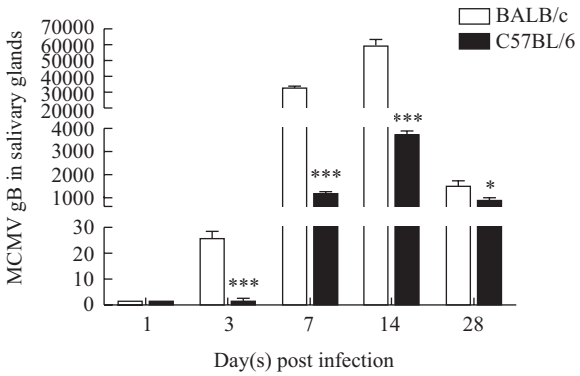

Fig. 4 Viral loads of salivary glands in BALB/c and C57BL/6 mice after MCMV infection
Viral replications in salivary glands and corresponding comparisons between the two mouse strains were assessed (*n*=3–5 each). Viral replications of infection groups were calculated by reference to MCMV gB levels on day 1 after normalizing to HPRT1. *P<0.05, ***P<0.001 *vs.* the corresponding group of BALB/c mice

low expression afterwards, along with limited levels of other AIM2 inflammasome components. This result was consistent with previous study[18]. The activation of AIM2 inflammasome started later in C57BL/6 mice than in BALB/c mice. Contents of AIM2 in C57BL/6

mice increased on day 3, but maintained a higher level till the day 14/28 post infection. Caspase-1 p20 and mature IL1β increased on day 7, 14 and 28, while expression of IL18 in serum showed a persistently high level with a double peak curve. Clearly, the activation status of AIM2 inflammasome was different in BALB/c and C57BL/6 mice. C57BL/6 mice held a longer duration of increased AIM2, which provided more caspase-1 p20 and finally produced abundant IL1β and IL18 to control MCMV infection effectively.

qRT-PCR was more sensitive than plaque assay for detecting viral replications[26]. Our study evaluated viral replications from day 1 to day 28 post infection. Because of the relatively long observation period, viral loads of infection groups might vary a lot. So we chose qRT-PCR to detect viral replications in salivary glands. Viral replications in salivary glands and pathological damage of livers further verified the defective antiviral responses in BALB/c mice. Considering the crucial function of AIM2 inflammasome in bridging innate and adaptive immunity, this outcome was probably caused by the shorter-lived activation of AIM2 inflammasome.

In our data, contents of AIM2 in PMs and spleens did not correspond to changes of downstream effectors in a point-to-point form during MCMV infection. Contents of IL18 in C57BL/6 mice started to increase on day 1 post infection, while AIM2 inflammasome had not yet been activated. The antiviral responses were complicated. We did not exclude the possibility that other inflammasomes were involved in this process, such as Nod-like Receptor Protein 3 (NLRP3) inflammasome. Li *et al* have found that NLRP3 can be activated in latent respiratory virus infection caused by MCMV[27]. But it was certain that the different activation status of AIM2 inflammasome did influence the infection outcomes. Further work needs to be done to elucidate the different infection outcomes in BALB/c and C57BL/6 mice. Through adopting AIM2 knock-out mice on BALB/c and C57BL/6 background and assessing them with the corresponding wild-type control mice, more factors in detail will be discovered.

Clinically, people with HCMV infection suffer different severities of clinical symptoms, excluding the influence of individual immunity. Our previous study has proved the positive function of AIM2 in facilitating the host immunity to HCMV[28]. It is natural to wonder whether the activation status of AIM2 inflammasome affects the disease progress of HCMV. Our study may provide clues to further researches.

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

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