Impaired NLRP3 inflammasome activation/pyroptosis leads to robust inflammatory cell death via caspase-8/RIPK3 during coronavirus infection

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Running title: GSDMD inhibits coronavirus-induced inflammatory cell death

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ABSTRACT

Coronaviruses have caused several zoonotic infections in the past two decades, leading to significant morbidity and mortality globally. Balanced regulation of cell death and inflammatory immune responses is essential to promote protection against coronavirus infection; however, the underlying mechanisms that control these processes remain to be resolved. Here we demonstrate that infection with the murine coronavirus mouse hepatitis virus (MHV) activated the NLRP3 inflammasome and inflammatory cell death in the form of PANoptosis. Deleting NLRP3 inflammasome components or the downstream cell death executioner gasdermin D (GSDMD) led to an initial reduction in cell death followed by a robust increase in the incidence of caspase-8- and receptor-interacting serine/threonine-protein kinase 3 (RIPK3)-mediated inflammatory cell death after coronavirus infection. Additionally, loss of GSDMD promoted robust NLRP3 inflammasome activation. Moreover, the amounts of some cytokines released during coronavirus infection were significantly altered due to the absence of GSDMD. Altogether our findings show that inflammatory cell death is induced by coronavirus infection and that impaired NLRP3 inflammasome function or pyroptosis can lead to negative consequences for the host. These findings may have important implications for studies of coronavirus-induced disease.

Coronaviruses are single-stranded positive sense RNA viruses with a wide range of hosts (1). Four coronavirus genera (alpha, beta, gamma, and delta) have been identified based on their genetic and serologic properties. Two members of the betacoronavirus genera, severe acute respiratory syndrome-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome-associated coronavirus (MERS-CoV), have caused zoonotic events in the recent past (2). Now, a new member of the betacoronavirus genera, severe acute respiratory syndrome-associated coronavirus (SARS-CoV-2), which was first isolated in Wuhan, China in 2019, has emerged and is causing a pandemic (3). SARS-CoV-2 infection leads to Coronavirus Disease 2019 (COVID-19), which is most often a mild to moderate respiratory illness with symptoms.
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including fever, fatigue, and dry cough (4). However, patients can develop severe COVID-19 with acute respiratory distress syndrome (ARDS) and present with acute lung damage (5–7).

Furthermore, cytokine storm has been suggested to be involved in the pathogenesis of severe and critical cases (8–10). Many cytokines, including proinflammatory cytokines and chemokines, have been found to be increased after SARS-CoV-2 infection (9, 11). Some of them, such as TNF and IL-6, are correlated with disease development (8, 9, 12). Despite these findings, little is known about what cellular and molecular mechanisms contribute to the lung damage and determine the onset of cytokine storm in response to SARS-CoV-2 infection.

With other infectious respiratory RNA viruses, such as influenza A virus (IAV) and MERS, studies have shown that aberrant programmed cell death may be a critical driver of organ failure and overt cytokine release in severe cases (13–16). Programmed cell death is a genetically encoded, actively controlled cellular process for the targeted removal of redundant, irreversibly damaged, and/or potentially deleterious cells (17). Among the programmed cell death pathways, apoptosis, pyroptosis, and necroptosis have been the best characterized and function during organismal development and infection. Apoptosis is a caspase-8/10– or -9– dependent form of cell death that is executed by caspase-3 and -7 (18, 19). Pyroptosis is executed by gasdermin family members (20), which can be activated through inflammasome activation-mediated caspase-1 cleavage of gasdermin D (GSDMD); caspase-11/4/5– or caspase-8– mediated cleavage of GSDMD; caspase-3– mediated cleavage of GSDME; or granzyme A– mediated cleavage of GSDMB (21–27). Necroptosis is characterized by RIPK3-activated MLKL oligomerization to mediate cell death (28). Numerous pathogens induce at least one of these three programmed cell death pathways during infection. Some pathogens, such as IAV and vesicular stomatitis virus (VSV), can even induce all three in the same cell population through a process termed PANoptosis (29, 30).

The concept of PANoptosis was established based on our studies that have identified crosstalk between the inflammasome/pyroptosis and apoptosis and necroptosis (30–41). PANoptosis ('P', Pyroptosis; 'A', Apoptosis; 'N', Necroptosis; and 'optosis', a form of programmed cell death) defines a unique inflammatory programmed cell death pathway distinct from pyroptosis, apoptosis, and necroptosis that is triggered in response to certain infections and cellular stresses and occurs downstream of a multiprotein complex called the PANoptosome (34, 37–39). This molecular scaffold contains machinery required for inflammasome activation/pyroptosis (such as NLRP3, ASC, caspase-1), apoptosis (caspase-8), and necroptosis (RIPK3/RIPK1) (34, 37–39).

The synergy and crosstalk between these molecules, which were previously thought to be dedicated to their representative cell death pathways, is critical to modulate several inflammatory and infectious diseases and cancer. PANoptosis can trigger the release of proinflammatory cytokines and damage-associated molecular patterns (DAMPs), which lead to robust inflammation (29, 42). Therefore, dysregulation of these pathways can be detrimental to the host during infection.

Sporadic studies have demonstrated that betacoronavirus proteins or infection with full virus can induce apoptosis, pyroptosis, and necroptosis in specific cell types. In HEK293T cells reconstituted with RIPK3 or inflammasome components, open reading frame-3a (ORF-3a) of SARS-CoV activates necroptosis (in RIPK3-reconstituted cells) (16) or caspase-1 (in inflammasome component-reconstituted cells) (16, 43), suggesting the capacity of SARS-CoV to activate pyroptosis. Furthermore, transfection assays indicate that ORF-8b (44) and ORF-3a (43) of SARS-CoV can activate the NLRP3 inflammasome in THP1 cells. In addition, the ion channel activity of the SARS-CoV envelope (E) protein has been shown to contribute to IL-1β release in vivo after SARS-CoV infection (45–47), suggesting an essential role for the E protein in regulating SARS-CoV–induced inflammasome activation. This role is further supported by work showing that transfection of the SARS-CoV E protein into Vero E6 cells reconstituted with inflammasome components leads to IL-1β release (48). Moreover, MERS and mouse hepatitis virus (MHV), another betacoronavirus, have also been shown to activate apoptosis (15, 49) and induce IL-1β
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release (50, 51), suggesting the activation of the inflammasome. However, the mechanistic details of the cell death induced by coronaviruses and the functional consequences of this cell death have not been elucidated.

Understanding how coronaviruses activate cell death and how host factors regulate coronavirus-induced cell death and proinflammatory cytokine expression is key to identifying effective treatment strategies. In this study, we used the mouse coronavirus MHV to systematically assess coronavirus-induced cell death. MHV is the prototypical laboratory coronavirus and has been used to guide our understanding of coronavirus immune responses (52). By infecting cells lacking one or more specific programmed cell death pathway, we evaluated the cell death and inflammatory cytokine release to examine the mechanistic details of these pathways. Our results highlight the role of cell death pathways in coronavirus infection.

Results
Coronavirus infection induces PANoptosis

To systematically investigate the programmed cell death pathways induced by coronavirus infection, we infected bone marrow-derived macrophages (BMDMs) from mice with strain A59 of MHV and monitored the effects on cell death pathways. We found that caspase-1 was cleaved after MHV infection, indicating that inflammasome activation was occurring (Figure 1A). Additionally, pyroptosis was induced by MHV infection, as evidenced by the presence of the p30 cleaved fragment of GSDMD (Figure 1A). Consistent with previous reports (49, 53), MHV infection also triggered apoptosis, as indicated by the cleavage of caspase-8, -7 and -3 (Figure 1A). Previous studies have shown that caspase-3 or -7 activation can inactivate GSDMD by processing it to produce a p20 fragment (54, 55). In line with this, a p20 band of GSDMD was observed in MHV-infected cells (Figure 1A). Moreover, phosphorylated MLKL was detected in MHV-infected cells, suggesting that MHV could also induce necroptosis. Together, these data indicate that, similar to what we have shown previously with IAV and VSV (29, 30), MHV can induce PANoptosis.

To monitor the kinetics of cell death in response to MHV infection, we used the nucleic acid stain SYTOX Green and investigated cell death in real-time. It is known that the spike protein of coronaviruses can mediate viral entry into infected cells and also induce syncytia during the infection (56–58). Syncytia formation was observed following MHV infection, and the number of dead cells gradually increased over time (Figures 1B and 1C). In addition, we also evaluated cytokine release from the infected cells. In line with the cleavage of caspase-1, which indicated inflammasome assembly, the downstream cytokines IL-1ß and IL-18 were detected, and their concentrations gradually increased in the supernatants of infected cells over time (Figure 1D). Other proinflammatory cytokines like IL-6 and TNF were also detected (Figure 1D), indicating that MHV infection can induce inflammatory responses. Taken together, these data show that coronavirus infection can initiate PANoptosis and trigger inflammatory responses.

Pyroptosis deficiency leads to increased cell death after coronavirus infection

In response to canonical inflammasome triggers, GSDMD is processed by caspase-1, resulting in the release of the N-terminus of GSDMD to form pores in the cell membrane (22, 25, 59, 60). The GSDMD pore can initiate pyroptotic cell death and also facilitates the secretion of active inflammasome-dependent cytokines IL-1ß and IL-18 (21, 61). To investigate the importance of inflammasome activation and GSDMD-mediated pyroptosis in MHV-induced cell death, we infected BMDMs deficient in caspase-1/11 or GSDMD. At 8 h post-infection, deletion of caspase-1/11 or GSDMD resulted in a decrease in cell death. Counterintuitively, though, deleting caspase-1/11 or GSDMD led to substantially increased cell death in response to MHV infection compared to the cell death in wild type (WT) cells at 14 h post-infection (Figure 2A). By contrast, cell death induced by IAV, another respiratory infectious RNA virus, or VSV, a single-stranded RNA virus, was not affected in BMDMs lacking caspase-1/11 or GSDMD early or later in infection (Figures 2B, 2C, S1A and S1B), suggesting that losing caspase-1/11 or GSDMD
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specifically affects coronavirus-induced cell death. In line with this, activation of caspase-8, -7, and -3 and phosphorylation of MLKL was greatly increased after MHV infection in cells lacking caspase-1/11 or GSDMD compared to their activation in WT cells (Figure 2D). These data suggest that without a functional inflammasome or GSDMD-mediated pyroptosis, MHV tends to induce more apoptosis and necroptosis. Of note, in spite of the robust cell death in caspase-1/11–deficient cells, no increase in the presence of the p30 fragment of GSDMD, which is responsible for executing pyroptosis, was detected after MHV infection (Figure 2D). These results indicate that the pyroptotic product of GSDMD is processed by caspase-1/11 during MHV infection at the timepoint tested.

In addition to GSDMD, other host factors are also able to mediate pore formation leading to cell death, including MLKL, which can form pores to initiate necroptosis (28), and GSDME, which can be activated by caspase-3 to induce pyroptosis (26, 62). To investigate whether these two pore-forming proteins function similarly to GSDMD in the context of coronavirus infections, we infected Mlkl<sup>−/−</sup> or Gsdme<sup>−/−</sup> BMDMs with MHV. We found a slight decrease in MHV-induced cell death in the Mlkl<sup>−/−</sup> or Gsdme<sup>−/−</sup> BMDMs compared with WT cells (Figure S2A). Moleculaly, we found that MLKL may play a role in promoting MHV-induced inflammasome assembly, as evidenced by reduced caspase-1 activation in the Mlkl<sup>−/−</sup> cells (Figure S2B). While apoptosis markers were comparable between WT and Mlkl<sup>−/−</sup> cells, the amount of the p30 fragment of GSDMD detected was reduced in Mlkl<sup>−/−</sup> cells, which is consistent with the levels of activated caspase-1, suggesting that activation of GSDMD-mediated pyroptosis during MHV infection depends on caspase-1 (Figure S2B). GSDME deficiency had no impact on GSDMD activation, and apoptosis and necroptosis were not impaired in GSDME-deficient cells compared with WT after MHV infection (Figure S2C).

Together, these data suggest that inflammasome activation and GSDMD-mediated pyroptosis play a critical role in inhibiting coronavirus-induced apoptosis and necroptosis, and loss of the inflammasome/GSDMD pathway results in robust cell death.

Loss of the NLRP3 inflammasome leads to increased apoptosis and necroptosis

Some coronavirus proteins and certain coronaviruses are known to initiate NLRP3 inflammasome assembly (43, 44, 50, 63). However, whether they can induce the activation of other inflammasomes has not been examined. To investigate this, we infected cells deficient in NLRP3, AIM2, NLRC4, or caspase-11 with MHV. We observed that NLRP3 deficiency completely abolished caspase-1 activation following MHV infection, while deleting AIM2, NLRC4, or caspase-11 did not impair MHV-induced caspase-1 activation (Figure 3A). Given that NLRP3 inflammasome activation requires a priming signal to upregulate the expression of NLRP3, we checked NLRP3 protein levels following MHV infection and found that NLRP3 expression was increased after MHV infection (Figure S3). It was previously shown that MyD88 is necessary for TNF and IL-6 release in response to MHV infection (64), suggesting that MyD88 is required to prime the inflammatory response. Consistent with this, cells lacking MyD88 showed impaired NLRP3 expression after MHV infection (Figure S3), indicating that MyD88 is essential for the priming of the MHV-induced NLRP3 inflammasome. These findings suggest that MHV infection specifically induces the NLRP3 inflammasome. The reduced caspase-1 cleavage was further supported by impaired GSDMD cleavage to form the p30 fragment in MHV-infected Nlrp3<sup>−/−</sup> cells (Figure 3A), suggesting pyroptosis was not executed. By contrast, AIM2<sup>−/−</sup>, NLRC4<sup>−/−</sup>, and caspase-11–deficient BMDMs showed similar pyroptotic GSDMD cleavage when compared with WT BMDMs (Figure 3A). These data indicate that at the timepoint tested, MHV-induced GSDMD activation depends on the NLRP3 inflammasome.

Given that both caspase-1/11 and NLRP3 are required for GSDMD cleavage, and caspase-1/11–deficiency led to increased cell death similar to that observed in GSDMD-deficient cells after MHV infection (Figure 2A), we investigated whether NLRP3 functions similarly to caspase-1 during MHV infection. We found that, similar to what we observed in caspase-1/11–deficient cells, the activation of caspase-8, -7, and -3 and MLKL was markedly increased in MHV-infected Nlrp3<sup>−/−</sup> cells compared with WT
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cells (Figure 3A). By contrast, MHV-induced apoptosis and necroptosis activation were not increased in \textit{Aim}^{2−/−}, \textit{Nlrp}^{4−/−}, or \textit{Casp}^{11−/−} cells (Figure 3A). In line with the cell death markers, quantitatively, more cell death was observed in MHV-infected \textit{Nlrp}^{3−/−} cells compared with WT cells at 14 h post-infection following the initial slight reduction at 8 h (Figures 3B, 3C and S2A). In the absence of AIM2, NLRC4, or caspase-11, MHV-induced cell death was not significantly affected (Figures 3B and 3C), further supporting the unique role of NLRP3 during coronavirus infection. We also noted that the increase in cell death corresponded to a decrease in MHV viral replication, as cells deficient in NLRP3, caspase-1, or GSDMD had less virus titers compared to that of WT cells (Figure S4).

To confirm the role of the other inflammasome components during MHV infection, we evaluated ASC− and caspase-1−deficient cells. Consistent with the function of NLRP3, deleting caspase-1 or ASC increased cell death at 14 h post-infection (Figure S2A). We consistently observed that there was more cell death in the WT cells at around 8 h post-infection than in cells lacking NLRP3, ASC, caspase-1, or GSDMD (Figures 3B, 3C, and S2A), suggesting that early cell death during MHV infection is largely caused by inflammasome-mediated pyroptosis.

Taken together, these data imply that coronaviruses specifically activate the NLRP3 inflammasome, which supports viral replication, and GSDMD activation during coronavirus infection depends on NLRP3 inflammasome assembly.

\textbf{Increased cell death in the absence of NLRP3/pyroptosis is dependent on caspase-8 and RIPK3}

We have previously shown that IAV- and VSV-induced PANoptosis activation completely (IAV) or heavily (VSV) depend on caspase-8 and RIPK3 (29, 30). Consistent with these previous results, we again observed IAV- and VSV-induced cell death were abolished or dramatically reduced, respectively, in \textit{Casp}^{8−/−}\textit{Ripk}^{3−/−} cells (Figures S1A and S1B). We then investigated whether \textit{Casp}^{8−/−}\textit{Ripk}^{3−/−} cells have a similar phenotype following MHV infection. While \textit{Casp}^{1−/−}\textit{Casp}^{11−/−} and \textit{Gsdmd}^{−/−} cells showed robust cell death at 14 h after MHV infection, RIPK3 deficiency slightly decreased MHV-induced cell death, and cell death in \textit{Casp}^{8−/−}\textit{Ripk}^{3−/−} cells was much less than that in WT cells (Figures 4A and 4B). Furthermore, deleting caspase-8 and RIPK3 can block the increase in cell death caused by the loss of caspase-1/11 in response to MHV infection (Figures 4A and 4B). These observations suggest that MHV-induced cell death largely depends on caspase-8 and RIPK3. Additionally, we also observed consistently increased activation of caspase-8, -7, and -3 and MLKL in cells deficient in caspase-1/11 or GSDMD, while there was little activation of the inflammasome (caspase-1), pyroptosis (GSDMD), apoptosis (caspase-8, -7, and -3), or necroptosis (pMLKL) detected in \textit{Casp}^{8−/−}\textit{Ripk}^{3−/−} cells (Figure 4C), further supporting the critical role of caspase-8 and RIPK3 in MHV-induced cell death. In sum, these data suggest that coronavirus-induced cell death predominantly signals through caspase-8 and RIPK3 together, whereas GSDMD activation can inhibit caspase-8– and RIPK3–mediated cell death.

\textbf{GSDMD deficiency affects cytokine release in response to MHV infection}

Given that the loss of GSDMD had remarkable effects on MHV-induced inflammatory cell death, we investigated whether this loss would also affect MHV-induced cytokine release. Consistent with the remarkably increased caspase-1 activation in \textit{Gsdmd}^{−/−} cells, IL-1β and IL-18 levels were significantly higher in \textit{Gsdmd}^{−/−} cells than in WT cells following MHV infection (Figures 5A and 5B), indicating that blocking GSDMD alone cannot suppress inflammasome-dependent cytokine release and instead promotes this release during coronavirus infection.

It has been recently suggested that cytokine storm contributes to the disease development during SARS-CoV-2 infection (10, 65), but little is known about how these cytokines become dysregulated in patients with severe or critical disease. Several cytokines including IL-6, TNF, G-CSF, GM-CSF, IP-10, and MIP-1α have been found to be increased following SARS-CoV-2 infection and correlate with COVID-19 development (8, 9, 12). We therefore examined whether deleting GSDMD would affect the...
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Discussion

In this study, we systematically investigated cell death and inflammasome activation and found that coronavirus infection activated pyroptosis, apoptosis, and necroptosis (PANoptosis) and specifically induced NLRP3 inflammasome assembly. Deleting components of the NLRP3 inflammasome, i.e. NLRP3, ASC, or caspase-1, resulted in a substantial increase in apoptosis and necroptosis beyond 8 h of infection. The increased cell death was likely a result of the loss of GSDMD activation, since cells lacking GSDMD showed a similar phenotype. Our data suggest that GSDMD inhibits coronavirus-induced cell death. The underlying mechanism by which GSDMD mediates the inhibition is unknown, but this phenotype is uncommon. In the case of the classical inflammasome triggers, deleting inflammasome components or GSDMD causes a delay and a reduction in cell death compared to the cell death in WT cells (22, 66, 67). Additionally, in the case of two other RNA viruses we tested, IAV and VSV, which are both known to activate PANoptosis, neither showed increased cell death in caspase-1/11− or GSDMD−deficient cells after infection. Within the gasdermin family, GSDME has also been shown to be critical in mediating pyroptosis in macrophages (54). However, deleting GSDME had little effect on the induction of coronavirus-induced PANoptosis. Similar results were observed with MLKL-deficient cells. These data indicate that GSDMD plays a unique role in addition to mediating pyroptosis during coronavirus infection. It was noteworthy that we observed a clear transition in cell death over the time course of MHV infection; NLRP3−, caspase-1/11−, or GSDMD−deficient cells showed much less cell death compared with WT cells at around 8 h post-infection, but then exhibited greatly elevated cell death at later times. These data suggest that early during infection, the coronavirus induced the activation of the NLRP3 inflammasome and GSDMD. The activated GSDMD then controlled the induction of other forms of coronavirus-induced cell death to prevent dysregulated cell death from occurring, and this dysregulated cell death was detrimental to viral replication.

The substantially increased IL-1β and IL-18 release in GSDMD-deficient cells showed that blocking GSDMD during coronavirus infection is unlikely to inhibit the release of these cytokines and may actually increase the release. Since MHV induced PANoptosis, it is likely that without GSDMD pores, IL-1β and IL-18 were released through the MLKL or GSDME pore or both. It will be interesting to investigate the IL-1β and IL-18 release in cells lacking all three of these pore-forming molecules after coronavirus infection.

Our study focuses on MHV, the prototypical laboratory coronavirus. MHV does not infect humans and has key differences from the human coronaviruses, including the host cell receptor used for invasion (68, 69); this difference between the murine virus and SARS-CoV-2 prevents SARS-CoV-2 from being studied in WT mouse models. However, MHV and other mammalian coronaviruses have been important in guiding our understanding of coronavirus immune responses, particularly in the context of emerging infections where immune responses are poorly understood (52). For example, shortly after the SARS-CoV outbreak, an inflammatory storm that was known to occur in response to MHV was also found in response to SARS-CoV (70), and now a similar phenomenon seems to occur with SARS-CoV-2 (10, 65, 71–73). The extensive history of studies with MHV has served as a key starting point for investigations of human coronaviruses, including the initial identification of the spike protein as a potential vaccine target (74). Therefore, it is important for future studies to extend our findings in MHV to the pathogenic human coronaviruses.
Severe forms of disease caused by the human coronaviruses, including SARS, MERS, and COVID-19, have been characterized by lung damage (5, 7, 75, 76) and cytokine storm (10, 65, 71–73). Additionally, the mortality rate of all three diseases is extremely high in people over the age of 65 compared with younger populations (77–79). It is possible that the elderly tend to have increased incidence of underlying medical conditions that contribute to this death rate. However, based on the observation that loss of NLRP3 resulted in increased cell death in the context of MHV, it will be important to extend these findings to human coronaviruses and determine whether compromised NLRP3 inflammasome function in the elderly could be a contributing factor. Older mice have impaired NLRP3 inflammasome activity in response to respiratory infectious agents, including *S. pneumoniae* and IAV (80, 81). Macrophages and dendritic cells isolated from these mice show attenuated expression of NLRP3, ASC, and caspase-1 compared to the expression in young mice, which could partially explain the decreased NLRP3 inflammasome activation in older mice. Additionally, older people have reduced NLRP3 expression compared to younger individuals in response to IAV infection (82). Given that loss of a functional NLRP3 inflammasome resulted in increased cell death and that loss of the downstream executioner GSDMD led to increased proinflammatory cytokine and chemokine release after MHV infection, it will be important to explore whether impaired NLRP3 inflammasome activation contributes to the high mortality rate of COVID-19 in the elderly population.

Overall, our study identified that coronavirus infection induced NLRP3 inflammasome assembly and activated pyroptosis, apoptosis, and necroptosis (PANoptosis). Furthermore, the inhibition of the NLRP3 inflammasome/pyroptosis may be detrimental to the host and result in increased cell death and inflammatory cytokine release in the context of coronaviruses. It is critical for future studies to extend our findings with MHV to human coronaviruses, particularly SARS-CoV-2, to understand the basic biological processes that occur during the infection and inform which treatments should proceed for clinical evaluation.

These findings here improve our understanding of coronavirus pathogenesis as it relates to cell death and cytokine release and may provide some constructive insight to inform studies of COVID-19.

**Experimental procedures**

**Mice**

Nlrp3<sup>−/−</sup> (83), Casp11<sup>−/−</sup> (84), Gsdmd<sup>−/−</sup> (85), Gsdme<sup>−/−</sup> (86), Mlkl<sup>−/−</sup> (87), Asc<sup>−/−</sup> (88), Nlrc4<sup>−/−</sup> (89), Aim2<sup>−/−</sup> (90), Ripk3<sup>−/−</sup> (91), Casp8<sup>−/−</sup>Ripk3<sup>−/−</sup> (92), Casp8<sup>−/−</sup>Ripk3<sup>−/−</sup>Casp1/11<sup>−/−</sup> (35), and MyD88<sup>−/−</sup> (93) mice have been described previously. All mice were bred at the Animal Resources Center at St. Jude Children’s Research Hospital and were backcrossed to the C57BL/6 background. Animal study protocols were approved by St. Jude Children’s Research Hospital committee on the care and use of animals.

**Bone marrow-derived macrophages (BMDMs)**

Primary BMDMs were cultured for 6 days in IMDM (Thermo Fisher Scientific, 12440-053) supplemented with 10% FBS (Biowest, S1620), 30% L929-conditioned medium, 1% non-essential amino acids (Thermo Fisher Scientific, 11140-050), and 1% penicillin and streptomycin (Thermo Fisher Scientific, 15070-063). Then BMDMs were seeded into 12-well plates at a density of 1 million cells per well and incubated overnight before use.

**Murine hepatitis virus (MHV) culture**

The murine hepatitis virus (A59 strain) was propagated in 17Cl-1 cells as previously described (94). Virus titer was measured by plaque assay in 17Cl-1 cells.

**Influenza A virus (IAV) culture**

The influenza A virus (A/Puerto Rico/8/34, H1N1 [PR8]) was rescued by reverse genetics as previously described (95). Virus titer was measured by plaque assay in 17Cl-1 cells.

**Vesicular stomatitis virus (VSV) culture**
The Indiana strain of vesicular stomatitis virus was propagated in Vero cells, and virus titer was measured by plaque assay in Vero cells.

**Cell stimulation/infection**
For MHV infection, BMDMs seeded in 12-well plates were infected at an MOI of 0.1 in DMEM plain media (Sigma, D6171). For IAV and VSV infection, cells were infected at an MOI of 20 or 1, respectively. After 2 h of incubation with each virus, cells were supplemented with 10% FBS and then incubated for the indicated time.

**Immunoblot analysis**
For caspase protein analysis, BMDMs were lysed with the supernatant using 50 μL caspase lysis buffer (1× protease inhibitors, 1× phosphatase inhibitors, 10% NP-40, and 25 mM DTT) followed by the addition of 125 μL 4× SDS loading buffer. For analysis of all other proteins, the supernatants were removed, and cells were washed once with PBS. Then BMDMs were lysed with RIPA buffer. Electrophoresis was utilized to separate proteins in 10%-12% polyacrylamide gels, after which proteins were transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 1 h at room temperature. All the primary antibodies were incubated overnight at 4°C, and secondary antibodies with HRP were incubated for 1 h at room temperature. Images were developed using a GE Amersham Imager 600. The antibodies used were as follows: anti-caspase-1 (AdipoGen, AG-20B-0042, 1:2000), anti-caspase-3 (Cell Signaling Technology [CST], #9662, 1:1000), anti-cleaved caspase-3 (CST, #9661, 1:1000), anti-caspase-7 (CST, #9492, 1:1000), anti-cleaved caspase-7 (CST, #9491, 1:1000), anti-caspase-8 (CST, #4927, 1:1000), anti-cleaved caspase-8 (CST, #8592, 1:1000), anti-pMLKL (CST, #37333, 1:1000), anti-GSDMD (Abcam, ab209845, 1:1000), anti-β-actin (Proteintech, 66009-1-IG, 1:5000), anti-GAPDH (CST, #5174, 1:1000) and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, anti-rabbit [111-035-047], 1:5000; anti-mouse [315-035-047], 1:5000).

**Real-time cell death analysis**
Real-time cell death analysis was conducted as previously described (32). In brief, BMDMs were seeded in 24-well plates (0.5 × 10⁶ cells/well) and infected with the indicated virus. After 2 h of incubation, 100 nM SYTOX Green (Thermo Fisher Scientific, S7020) was added to the cells together with FBS. Images were analyzed using IncuCyte S3 software.

**Growth kinetics of MHV**
BMDMs were seeded in 24-well plates (0.5 × 10⁶ cells/well) and infected with MHV at an MOI of 0.1. After 2 h of incubation, virus was removed, and cells were washed twice with PBS. Then fresh media was added into each well, and supernatants were collected at the indicated time points. Viral titers were determined by plaque assay using 17Cl-1 cells.

**Cytokine analysis**
Cytokines were analyzed by multiplex ELISA (Millipore, MCYTOMAG-70K) or IL-18 ELISA (Invitrogen, BMS618-3) according to the manufacturer’s instructions.

**Statistical analysis**
GraphPad Prism 7.0 software was used for data analysis. Data are presented as mean ± SEM. The one-way ANOVA with Dunnett’s multiple comparisons test was used to determine the statistical significance. P values less than 0.05 were considered statistically significant where *P* < 0.05, **P** < 0.01, ***P** < 0.001, and ****P** < 0.0001.
Data availability: All data generated for this study are included within this manuscript.

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Competing financial interests
The authors declare no competing financial interests.

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GSDMD inhibits coronavirus-induced inflammatory cell death

Figure 1: Coronavirus infection induces PANoptosis (pyroptosis, apoptosis, and necroptosis)

A) Immunoblot analysis of pro- (p45) and cleaved caspase-1 (p20; CASP1), pro- (p53), activated (p30), and inactivated (p20) gasdermin D (GSDMD), pro- (p55) and cleaved caspase-8 (p18; CASP8), pro- (p35) and cleaved caspase-7 (p19; CASP7), pro- (p35) and cleaved caspase-3 (p17; CASP3), and phosphorylated MLKL (pMLKL) in bone marrow-derived macrophages (BMDMs) after murine hepatitis virus (MHV) infection for 12 h. Actin was used as the internal control.

B) Real-time analysis of cell death in BMDMs using the IncuCyte imaging system and SYTOX Green nucleic acid staining after infection with MHV. Results at the indicated timepoints are shown. The red denotes the dead cells counted during the analysis. Scale bar, 50 μm.

C) Quantification of the cell death observed in (B).

D) IL-1β, IL-18, IL-6, and TNF release from BMDMs infected with MHV at the indicated timepoints. Data are representative of at least three independent experiments. Data are shown as mean ± SEM (C and D). ns, not significant; *P < 0.05; ***P < 0.001; and ****P < 0.0001 (one-way ANOVA).
GSDMD inhibits coronavirus-induced inflammatory cell death

Figure 2: Pyroptosis deficiency leads to increased cell death after coronavirus infection

A, Real-time analysis of cell death in bone marrow-derived macrophages (BMDMs) using the IncuCyte imaging system and SYTOX Green nucleic acid staining after infection with murine hepatitis virus (MHV). Quantification (left) and images (right) of the cell death at the indicated timepoints are shown. The red in cell death images denotes the dead cells counted during the analysis. Scale bar, 50 μm. B and C, Real-time analysis of cell death in BMDMs using the IncuCyte imaging system and SYTOX Green nucleic acid staining after infection with influenza A virus (IAV) (B) or vesicular stomatitis virus (VSV) (C).

D, Immunoblot analysis of pro- (p45) and cleaved caspase-1 (p20; CASP1), pro- (p35), activated (p30), and inactivated (p20) gadermin D (GSDMD), pro- (p55) and cleaved caspase-8 (p18; CASP8), pro- (p35) and cleaved caspase-7 (p19; CASP7), pro- (p35) and cleaved caspase-3 (p17; CASP3), and phosphorylated MLKL (pMLKL) in BMDMs after MHV infection for 12 h. GAPDH was used as the internal control. Data are shown as mean ± SEM (A–C). ns, not significant; *P < 0.05; and ****P < 0.0001 (one-way ANOVA). Data are representative of at least three independent experiments.
Figure 3: Loss of the NLRP3 inflammasome leads to increased apoptosis and necroptosis

A, Immunoblot analysis of pro- (p45) and cleaved caspase-1 (p20; CASP1), pro- (p53), activated (p30), and inactivated (p20) gasdermin D (GSDMD), pro- (p55) and cleaved caspase-8 (p18; CASP8), pro- (p35) and cleaved caspase-7 (p19; CASP7), pro- (p35) and cleaved caspase-3 (p17; CASP3), and phosphorylated MLKL (pMLKL) in bone marrow-derived macrophages (BMDMs) after murine hepatitis virus (MHV) infection for 12 h. GAPDH was used as the internal control.

B, Real-time analysis of cell death in BMDMs using the IncuCyte imaging system and SYTOX Green nucleic acid staining after infection with MHV. Quantification of the cell death at the indicated timepoints is shown.

C, Representative cell death images from (B) are shown. The red denotes the dead cells counted during the analysis. Scale bar, 50 μm. Data are shown as mean ± SEM (B). Significant differences compared to WT are denoted as ***P < 0.001 and ****P < 0.0001 (one-way ANOVA). Data are representative of three independent experiments.
Figure 4: Increased cell death in the absence of NLRP3/pyroptosis is dependent on caspase-8 and RIPK3

A. Real-time analysis of cell death in bone marrow-derived macrophages (BMDMs) using the IncuCyte imaging system and SYTOX Green nucleic acid staining after infection with murine hepatitis virus (MHV). Quantification of the cell death at the indicated timepoints is shown. **B.** Representative cell death images from (A) are shown. The red denotes the dead cells counted during the analysis. Scale bar, 50 μm. **C.** Immunoblot analysis of pro- (p45) and cleaved caspase-1 (p20; CASP1), pro- (p53), activated (p30), and inactivated (p20) gasdermin D (GSDMD), pro- (p55) and cleaved caspase-8 (p18; CASP8), pro- (p35) and cleaved caspase-7 (p19; CASP7), pro- (p35) and cleaved caspase-3 (p17; CASP3), and phosphorylated MLKL (pMLKL) in BMDMs after MHV infection for 12 h. GAPDH was used as the internal control. Data are shown as mean ± SEM (A). Significant differences compared to WT are denoted as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 (one-way ANOVA). Data are representative of at least three independent experiments.
GSDMD inhibits coronavirus-induced inflammatory cell death

Figure 5: GSDMD deficiency leads to increased cytokine release after MHV infection
IL-1β (A), IL-18 (B), IL-6 (C), TNF (D), G-CSF (E), GM-CSF (F), IP-10 (G), and MIP-1α (H) release from bone marrow-derived macrophages (BMDMs) infected with murine hepatitis virus (MHV) at the indicated timepoints. *P < 0.05, **P < 0.01, and ****P < 0.0001 (one-way ANOVA). Data are representative of three independent experiments. Data are shown as mean ± SEM (A–H).
Impaired NLRP3 inflammasome activation/pyroptosis leads to robust inflammatory cell death via caspase-8/RIPK3 during coronavirus infection

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