

Role of the inflammasome-related cytokines IL-1 and IL-18 during infection with murine coronavirus

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Abstract The inflammasome, a cytosolic protein complex that mediates the processing and secretion of pro-inflammatory cytokines, is one of the first responders during viral infection. The cytokines secreted following inflammasome activation, which include IL-1 and IL-18, regulate cells of both the innate and adaptive immune system, guiding the subsequent immune responses. In this study, we used murine coronavirus, mouse hepatitis virus (MHV), infection of the central nervous system and liver to assess of the role of the inflammasome and its related cytokines on pathogenesis and host defense during viral infection. Mice lacking all inflammasome signaling due to the absence of caspase-1 and -11 were more vulnerable to infection, with poor survival and elevated viral replication compared to wild-type mice. Mice lacking IL-1 signaling experienced elevated viral replication but similar survival compared to wild-type controls. In the absence of IL-18, mice had elevated viral replication and poor survival, and this protective effect of IL-18 was found to be due to promotion of interferon gamma production in $\alpha\beta$ T cells. These data suggest that inflammasome signaling is largely protective during murine coronavirus infection, in large part due to the pro-inflammatory effects of IL-18.

Keywords Inflammasome · IL-18 · IL-1 · Murine coronavirus

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Importance

Multicellular organisms are constantly exposed to microorganisms such as viruses and bacteria, many of which are pathogenic. When a pathogenic microorganism first infects a host, some of the first elements of the immune system that it encounters are innate inflammatory pathways, which use receptors to detect conserved pathogen-associated molecular patterns and trigger production of proinflammatory cytokines, proteins that regulate subsequent elements of the immune response. One of these innate inflammatory pathways is the inflammasome, which includes a large variety of sensors that lead to secretion of several cytokines, including IL-1 and IL-18. To better understand the roles of the inflammasome and its related cytokines, we infected mice deficient in one of the several inflammasome components with murine coronavirus and observed the subsequent changes in disease course and immune response. We show that the inflammasome as a whole is protective, while IL-1 has a minimal role in host defense. IL-18 is shown to be protective, and the mechanism of this protection is determined to be promotion of interferon gamma production by $\alpha\beta$ T cells.

Introduction

During infections, pathogens first encounter the innate immune system, which includes germline encoded pattern recognition receptors (PRR) that detect conserved pathogen-associated molecular patterns (PAMP) and induce production of pro-inflammatory cytokines and chemokines. These messengers, which include type 1 interferon, IL-6, TNF α , and others, can have direct anti-pathogen effects and help induce and guide the subsequent components of the innate immune response as well as the adaptive immune response. Innate inflammatory

pathways are therefore key regulators of much of the host response to pathogens. Many pathways can also drive damaging pathological effects when improperly regulated; so, studying these pathways can aid in both treatment of inflammatory diseases and improvement of host responses to pathogens.

One innate inflammatory pathway is the inflammasome. The inflammasome is a multimeric protein complex that forms in the cytosol following detection of a diverse range of ligands, and which is responsible for activation of caspase-1 (Martinon et al. 2002). Caspase-1 in turn processes members of the IL-1 family of cytokines into their active forms (Howard et al. 1991; Thornberry et al. 1992; Gu et al. 1997; Ghayur et al. 1997) leading to their secretion. These cytokines, including IL-1 α , IL-1 β , and IL-18, are pro-inflammatory and induce a variety of immunomodulatory effects, capable of leading to both host protection and damaging pathological response. IL-1 α and IL-1 β induce recruitment of neutrophils (Lee et al. 2015), polarize T cells to adopt a Th17 phenotype (Chung et al. 2009), and promote DC activation for priming (Ichinohe et al. 2009; Ramos et al. 2012). IL-18 signaling promotes production of interferon gamma (Okamura et al. 1995; Kohno et al. 1997; Bellora et al. 2012; Serti et al. 2014) and cytotoxicity (Okamura et al. 1995; Dao et al. 1996; Tsutsui et al. 1996) in a variety of cell types. Characterization of the role of the inflammasome and its related cytokines during viral infection is ongoing. While it has been shown that a viroporin protein of the severe acute respiratory syndrome (SARS) coronavirus activates the NLRP3 inflammasome, possibly contributing to disease severity (Nieto-Torres et al. 2015) and that inflammasome activation enhances pathology in a murine model of fulminant hepatitis (Guo et al. 2015), little other data are available regarding inflammasome activation in the context of coronavirus infection.

Murine coronavirus, mouse hepatitis virus strain A59 (MHV) is a positive strand RNA virus that provides a tractable model for studying pathogenesis of and host responses to coronaviruses. It has a wide organ tropism and is capable of infecting the brain, spinal cord, liver, spleen, lungs, and other organs. Infection can cause hepatitis in the liver and encephalitis and meningitis in the CNS. Following viral clearance, mice develop a demyelinating disease with phenotypic similarities to multiple sclerosis (MS) (Weiss and Leibowitz 2011). MHV can therefore be used to model a variety of pathologies.

MHV infection induces a strong innate inflammatory response, which includes induction of type 1 interferon (Roth-Cross et al. 2008), TNF α , IL-6, and IL-12 (Pearce et al. 1994; Parra et al. 1997; Rempel et al. 2004; Rempel et al. 2005), as well as neutrophil, macrophage, and NK infiltration of infected sites (Bergmann et al. 1999; Zhou et al. 2003; Iacono et al. 2006). Viral clearance is reliant on the interferon response (Zalinger et al. 2015) as well as interferon gamma production by CD4 and CD8 T cells and perforin-mediated cytotoxicity by CD8 T cells (Lin et al. 1997; Bergmann et al. 1999;

Ramakrishna et al. 2004; Iacono et al. 2006). Therefore, MHV is a useful model for studying the role of inflammatory pathways on the regulation of many aspects of both the innate and adaptive immune response.

In this study, we systematically examined the role of the inflammasome as a whole as well as the specific contributions of the inflammasome-related cytokines IL-1 and IL-18 to disease course and immune response during MHV infection. We demonstrate a protective role for the inflammasome as a whole and a role in control of viral replication but not survival for IL-1 signaling. IL-18 was found to be critically important for host defense, with poor survival and elevated viral replication in mice lacking IL-18 signaling. IL-18 signaling was demonstrated to promote interferon gamma production in activated T cell populations and to be crucial for global interferon gamma responses, and we therefore conclude that the inflammasome-related protein IL-18 improves host defense through the promotion of interferon gamma production in T cells.

Results

Caspase-1/11^{-/-} mice have heightened susceptibility to MHV infection

To determine the role of inflammasome signaling during murine coronavirus infection, we used a well-characterized model of intracranially (i.c.) inoculated MHV. Wild-type C57Bl/6 (WT) or C57Bl/6 mice lacking caspase-1 and -11 (Casp-1/11^{-/-}) were challenged with 5000 plaque forming units (PFU) of MHV, inoculated i.c., and survival was monitored. Casp-1/11^{-/-} mice were found to be acutely vulnerable to this infection, with roughly 40% survival compared to 90% survival among WT mice (Fig. 1a). These data demonstrate that inflammasome signaling is critical to host defense during MHV infection. To better understand what role the inflammasome plays in host defense, the magnitude of viral replication in infected organs was assessed. Mice were inoculated i.c. with 500 PFU of MHV, a nonlethal dose chosen to allow mice to survive the length of the experiment. Three, five, and seven days after infection mice were euthanized and the magnitude of viral replication in the brain, spinal cord, liver, and spleen was determined by plaque assay (Fig. 1b). Replication in the brain and spinal cord were the same in Casp-1/11^{-/-} and WT mice, suggesting that overall inflammasome signaling is not relevant for control of infection within the CNS. Viral replication was elevated in the liver 5 days after infection, and in the spleen, 3 and 5 days after infection in the Casp-1/11^{-/-} relative to WT, suggesting that infection of peripheral organs may be partially controlled by inflammasome signaling.

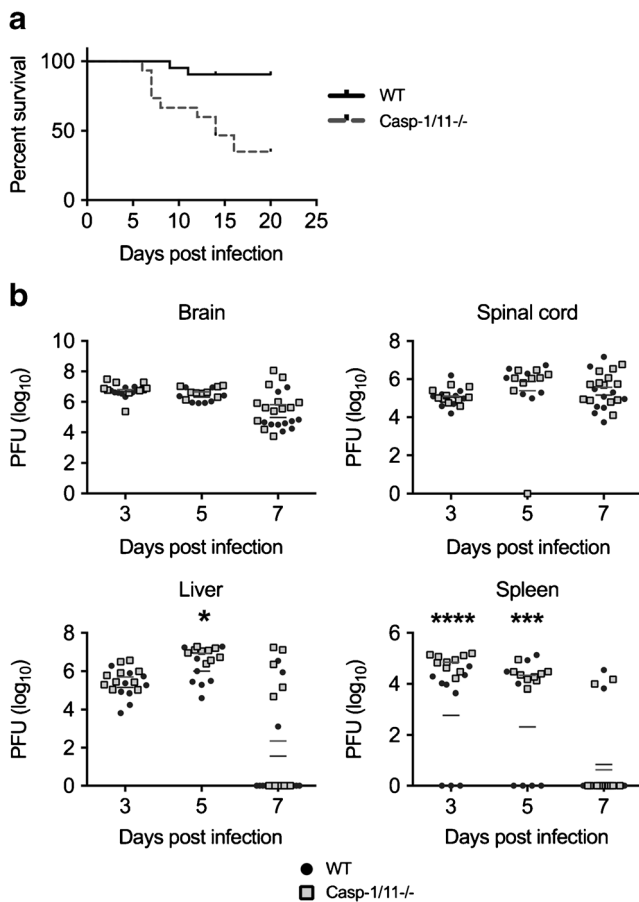


Fig. 1 Caspase-1/11^{-/-} mice are more susceptible to MHV infection than WT mice. **a** WT and Casp-1/11^{-/-} mice were inoculated i.c. with 5000 PFU of MHV, and survival was monitored. Data were pooled from two independent experiments, and mortality in the two genotypes is significantly different as determined by the Mantel-Cox and the Gehan-Breslow-Wilcoxon tests. **b** WT and Casp-1/11^{-/-} mice were inoculated i.c. with 500 PFU of MHV. Three, five, and seven days post-infection mice were euthanized and viral loads in their brains, spinal cords, spleens, and livers were assessed by plaque assay. Data were pooled from two independent experiments. Statistical significance was determined by a two-part test using a conditional *t* test and proportion test (**P* < 0.05; ****P* < 0.001)

IL-18 signaling controls viral replication and improves survival

Caspase-1 and -11 cleave a variety of substrates, the best characterized of which are IL-1 α , IL-1 β , and IL-18. IL-18 has been shown to promote production of interferon gamma and perforin in a variety of cells types (Okamura et al. 1995; Dao et al. 1996; Tsutsui et al. 1996; Kohno et al. 1997; Bellora et al. 2012; Serti et al. 2014), both of which are important for host defense against MHV infection (Lin et al. 1997; Parra et al. 1999). IL-18 signaling is partially redundant with IL-12 signaling, and the ability of each cytokine to compensate for the loss of the other varies among infection models. Therefore, while it is known that the downstream effects of IL-18 signaling, production of interferon gamma

and perforin, contribute to host defense during MHV infection, it is unknown if IL-18 is required.

While the poor survival of Casp-1/11^{-/-} mice could be due to a protective role of IL-18 signaling, the inflammasome also processes other cytokines. To determine the role of IL-18 specifically, we infected IL-18 receptor knockout (IL-18R^{-/-}) as well as WT mice with 500 PFU of MHV, a dose that is usually sublethal in WT mice, and monitored their survival (Fig. 2a). While roughly 90% of WT mice survived the infection, only 10% of IL-18R^{-/-} survived, demonstrating a critical role for IL-18 signaling in host defense. Please note that this dose is 90% smaller than the dose used to compare survival in WT and caspase-1/11^{-/-} mice. The experiment was not repeated at this higher dose due to the poor survival of IL-18R^{-/-} mice at the lower dose.

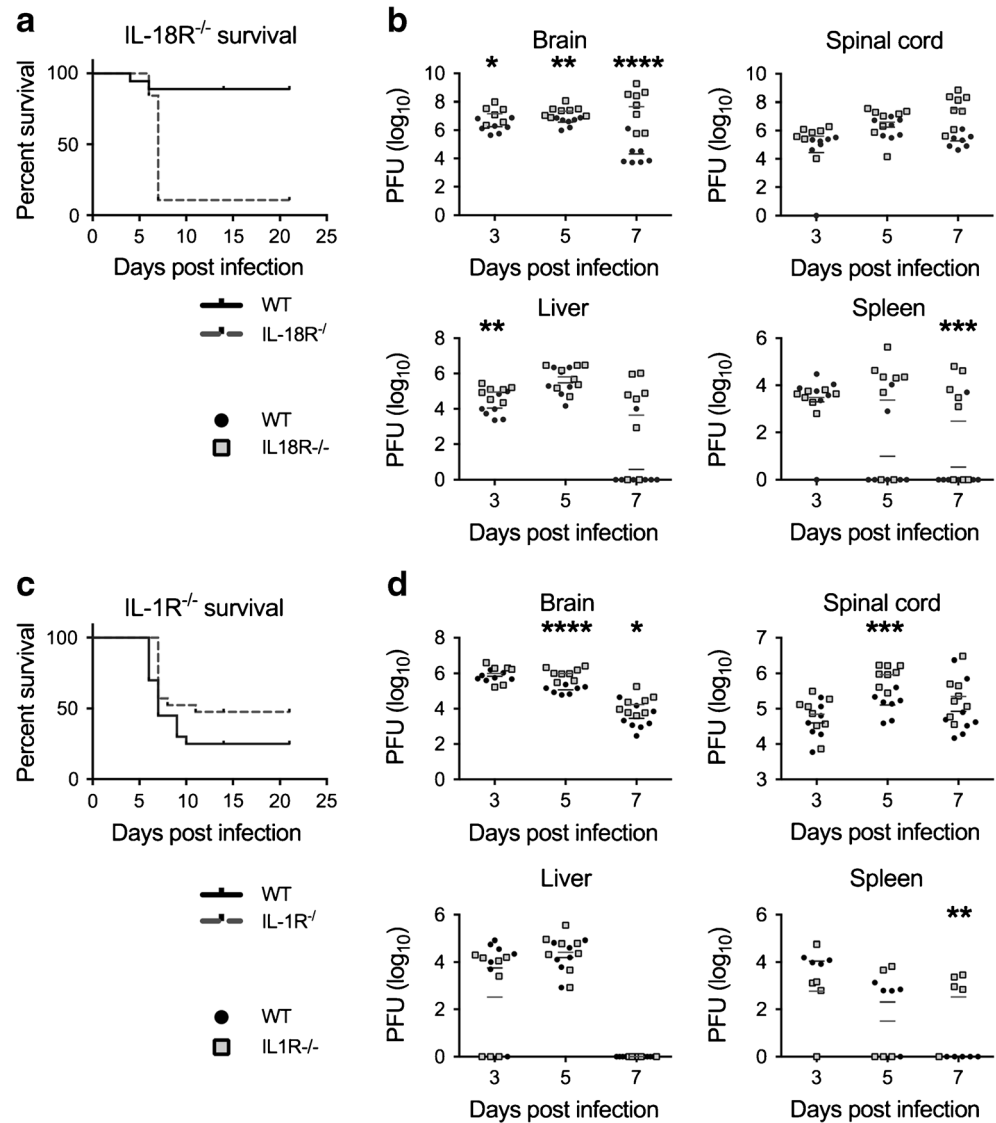
The impact of IL-18 signaling on replication of the virus was also assessed. WT and IL-18R^{-/-} mice were infected with 500 PFU of MHV. A randomly determined portion of the mice were euthanized 3, 5, or 7 days after infection and the viral load in their livers, spleens, brains, and spinal cords was determined by plaque assay (Fig. 2b). Viral load was elevated in IL-18R^{-/-} mice compared to WT mice at early time points in the liver, and there was a slight trend toward poor clearance at later time points in both the spleen and liver. Replication was elevated in the brains of IL-18R^{-/-} mice compared to WT mice at all tested time points, with the difference between IL-18R^{-/-} and WT mice growing larger at 7 days after infection, when WT mice began clearing virus. These data suggest that the lack of IL-18 signaling produces some immunodeficiency that limits the ability to clear the virus.

IL-1 signaling promotes control of viral infection but has no effect on survival

IL-1 α and IL-1 β are also caspase-1 substrates, and the susceptibility of Casp-1/11^{-/-} mice could be due in part to the loss of IL-1 signaling as well. Although IL-1 α and IL-1 β can have different effects, both signal through the IL-1 receptor. We therefore employed IL-1 receptor knockout (IL-1R^{-/-}) mice to assess the role of IL-1 signaling during MHV infection. These mice lack both IL-1 α and IL-1 β signaling. Research has shown that IL-1 promoted Th17 T cell response (Chung et al. 2009), while other studies have shown that it can also promote activation of DC during viral infection, even when the subsequent T cell response adopts a Th1 phenotype (Ichinohe et al. 2009; Ramos et al. 2012).

To assess the role of IL-1 signaling, we infected IL-1R^{-/-} mice with 5000 PFU of MHV and observed survival. Roughly 25% of WT mice survived the infection, while 50% of IL-1R^{-/-} mice did (Fig. 2c), although this difference is not statistically significant by either the Mantel-Cox or Gehan-Breslow-Wilcoxon tests. Viral load was tested in the liver, spleen, brain, and spinal cord by plaque assay (Fig. 2d). IL-1R^{-/-} mice had

Fig. 2 IL-18R^{-/-} mice are more susceptible to MHV infection compared to WT mice while IL-18R^{-/-} mice have increased survival but elevated viral loads. **a** WT and IL-18R^{-/-} mice were inoculated i.c. with 500 PFU of MHV or **c** WT and IL-1R^{-/-} were inoculated i.c. with 5000 PFU of MHV and survival was monitored. **b** WT and IL-18R^{-/-} mice or **d** WT and IL-1R^{-/-} mice were inoculated i.c. with 500 PFU of MHV. Three, five, and seven days post-infection mice were euthanized and viral loads in the brains, spinal cords, spleens, and livers were assessed by plaque assay. Data are pooled from two independent experiments for each genotype, with the exception IL-1R^{-/-} spleen titers, which were from one experiment. Survival in WT and knockout mice is significantly different as determined by the Mantel-Cox and the Gehan-Breslow-Wilcoxon tests in panel **a** but not in panel **c**. Statistical significance for panels **b** and **d** was determined by a two-part test using a conditional *t* test and proportion test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001)



similar viral loads in the liver compared to WT mice, but had a clearance defect in the spleen, and slightly elevated loads in the brain and spinal cord. These data indicate that IL-1 signaling provides some control of viral replication. The effect on survival is minimal, however, and suggests a minimal role for IL-1. This in turn suggests that viral load and survival are not necessarily correlated in this model.

The majority of IL-18 secretion is caspase-1/11 dependent

Lethality was higher among IL-18R^{-/-} mice than Casp-1/11^{-/-} mice (Figs. 1a and 2a), even though the IL-18R^{-/-} mice received one-tenth the dose of Casp-1/11^{-/-} mice. If caspase-1 is required for activation of IL-18, we would expect that Casp-1/11^{-/-} and IL-18R^{-/-} mice would phenocopy each other. However, our findings show that mice lacking IL-18 signaling are more susceptible to infection than those lacking caspase-1.

We propose two hypotheses to explain this difference. First, caspase-1 processes numerous substrates (Garlanda et al. 2013), and it is possible that one of these other substrates has a deleterious effect on animals during MHV infection. Therefore, its loss in the Casp-1/11^{-/-} mice would improve survival somewhat, partially countering the loss of IL-18 signaling. Our data suggests that IL-1 may be a candidate for this hypothesis, as survival was slightly higher in wild-type mice than IL-1R^{-/-} mice (Fig. 2c).

Alternatively, inflammasome-independent sources of IL-18 (Sugawara et al. 2001; Omoto et al. 2006; Omoto et al. 2010) may provide partial protection to the Casp-1/11^{-/-} mice. IL-18R^{-/-} mice lack all signaling regardless of the source of IL-18, depriving them of this partial protection. To address this hypothesis, we isolated serum from infected WT and Casp-1/11^{-/-} mice and determined the concentration of IL-18. While Casp-1/11^{-/-} mice had significantly

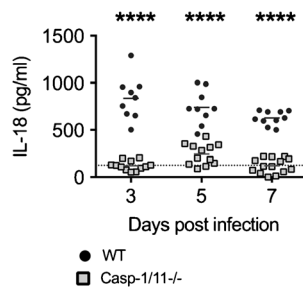


Fig. 3 IL-18 concentration is lower in the serum of Casp-1/11^{-/-} mice compared to WT mice. WT and Casp-1/11^{-/-} mice were infected with 500 PFU of MHV and serum was collected 3, 5, and 7 days post-infection. The concentration of IL-18 in the serum was determined by ELISA. Data are pooled from two independent experiments. Statistical significance was determined by a two-part test using a conditional *t* test and proportion test (****P* < 0.001)

lower levels of IL-18, a limited amount was observed 5 days after infection (Fig. 3). This low level of the cytokine may provide limited protection.

IL-18 signaling promotes interferon gamma production at the peak of infection

IL-18R^{-/-} mice are highly susceptible to MHV infection, with poor survival (Fig. 2a) and poor control of viral replication (Fig. 2c). We next sought to determine why the loss of IL-18 signaling leads to this phenotype. IL-18 acts on T cells, NK cells, and NKT cells to promote interferon gamma production (Okamura et al. 1995; Nagarajan and Kronenberg 2007; Bellora et al. 2012; Serti et al. 2014) as well as cytotoxicity, often through promoting perforin production (Okamura et al. 1995; Dao et al. 1996; Tsutsui et al. 1996). Both interferon gamma and perforin-mediated cell killing are important for host defense during MHV infection (Lin et al. 1997; Bergmann et al. 1999; Martinon et al. 2002; Bergmann et al. 2003; Ramakrishna et al. 2004); so, we hypothesized that in the absence of IL-18 signaling, mice develop an immunodeficiency that makes them vulnerable to MHV infection. We began by determining the concentration of interferon gamma in the serum of infected WT and IL-18R^{-/-} mice, hypothesizing that if one or more cell types produce interferon gamma in an IL-18-dependent manner during MHV infection, we might be able to observe a global decrease in interferon gamma production. Mice were infected with 500 PFU of MHV, and 3, 5, and 7 days after infection serum was isolated. Five days after infection, at the peak of interferon gamma production and viral titers, IL-18R^{-/-} mice had significantly lower cytokine concentrations (Fig. 4), despite slightly higher viral loads in several organs. This suggests that IL-18R^{-/-} mice have a defect in interferon gamma production. By 7 days after infection, higher interferon gamma levels are observed in IL-18R^{-/-} mice, which we speculate to be due to IL-18-independent sources reacting to elevated viral loads in the knockout mice compared to

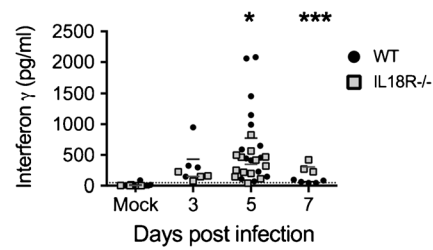


Fig. 4 Interferon gamma in serum of WT and IL-18R^{-/-} mice. WT and IL-18R^{-/-} mice were inoculated i.c. with 500 PFU of MHV. Three, five, and seven days after infection, serum was isolated and concentration of interferon gamma was assessed by ELISA. Data is pooled from two (for days 3 and 7) or four (for day 5) independent experiments. Statistical significance was determined by a two-part test using a conditional *t* test and proportion test (**P* < 0.05; ****P* < 0.001)

WT mice. This IL-18-independent production of interferon gamma may be insufficient to rescue these mice this late in the infection.

Interferon gamma production by splenic, but not liver cells, is dependent on IL-18 signaling

The IL-18-dependent defect of interferon gamma in the serum could be due to poor production by cells from all organs, or by cells from specific sites of infection. To assess this, we inoculated mice i.c. with 500 PFU of MHV, and 5 days later, at the peak of interferon gamma production (Fig. 4) and viral replication (Fig. 2), isolated cells from their spleens and livers. These cells were cultured in media with no stimulation overnight, and interferon gamma concentration in the culture supernatants was determined by ELISA. We found that interferon gamma production by cells from the liver was independent of IL-18 signaling while production by cells from the spleen was dependent on IL-18 signaling (Fig. 5). This may suggest an organ-specific role for IL-18 signaling.

Interferon gamma production by αβ T cells is partially dependent on IL-18 signaling by 7 days after infection

T cells have been shown to be critical for host defense during MHV infection (Howard et al. 1991; Thornberry et al. 1992; Gu et al. 1997; Ghayur et al. 1997; Bergmann et al. 2004; Phares et al. 2012), and IL-18 can drive T cell production of interferon gamma and cytotoxicity, so we tested the activation status of T cells from the spleen following MHV infection to determine if they exhibited an IL-18-dependent defect. The spleen was chosen because previous experiments (Fig. 5) indicated a spleen-specific defect. The T cell response is not expected to be fully developed by 5 days after MHV infection, so we isolated cells 7 days after infection at the peak of the T cell response. WT and IL-18R^{-/-} mice were infected with 500 PFU MHV, and cells were isolated from the spleen 7 days later. Splenocytes were incubated with MHV peptides S598 and M133, the known immunodominant MHV peptides (Xue

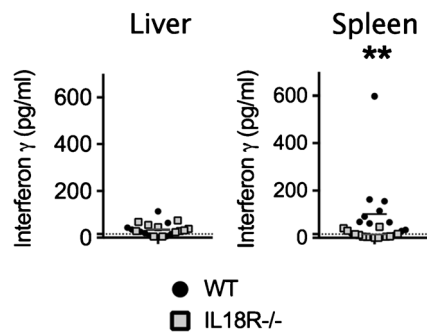


Fig. 5 Interferon gamma production is IL-18-independent in cells from the liver but IL-18-dependent in cells from the spleen. WT and IL-18R^{-/-} mice were inoculated i.c. with 500 PFU of MHV and 5 days later cells isolated from the spleen and liver. Cells were seeded into plates and incubated for 16 h, and interferon gamma concentration of the culture supernatant was determined by ELISA. Data are pooled from two independent experiments. Statistical significance was determined by a two-part test using a conditional *t* test and proportion test (***P* < 0.01)

et al. 1995; Castro and Perlman 1995; Lee et al. 2015), and brefeldin A, then phenotyped by flow cytometry. To assess the activation status of the T cell response, the percent (Fig. 6a) and total number (Fig. 6b) of CD4 and CD8 T cells expressing elevated levels of the activation markers CD44 and CD11a were quantified. All subtypes showed similar activation status between cells from WT and IL-18R^{-/-} mice.

The interferon gamma production of T cell populations was also assessed. CD4 and CD8 T cells with elevated surface expression of CD44⁺ or CD11a⁺ were gated on, and the percent (Fig. 7b) and total number (Fig. 7c) of these cells with elevated levels of interferon gamma were quantified. Production of interferon gamma was similar between cells from WT and IL-18R^{-/-} mice with the exception of two subsets. The percent of CD44^{high} CD4 T cells with elevated interferon gamma levels was higher in cells from WT mice compared to those from IL-18R^{-/-} mice, and the number of CD11a^{high} CD8 T cells with elevated interferon gamma levels was higher in WT than IL-18R^{-/-} mice. Although serum concentrations of interferon gamma are higher in IL-18R^{-/-} mice at this time point, it is possible that production of this cytokine by other cell types in response to the elevated viral loads is masking defective production by T cells, and that the reduction of interferon gamma production by T cells in IL-18R^{-/-} mice could lead to the poor survival and elevated viral replication observed in these mice.

Discussion

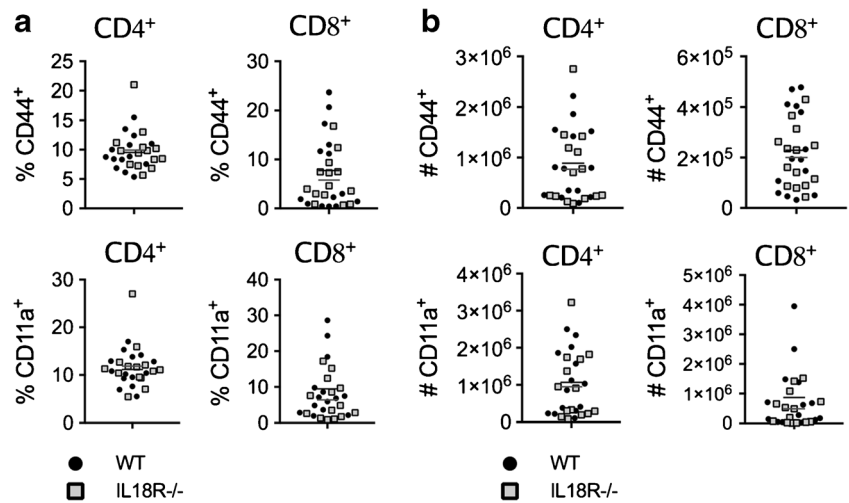
We have demonstrated a complex role for the inflammasome in host defense during murine coronavirus infection. Although mice lacking caspase-1 and caspase-11, and therefore both the canonical and non-canonical inflammasomes, are more vulnerable to MHV infection (Fig. 1), examination of the

individual roles of two inflammasome-related cytokines, IL-1 and IL-18, demonstrated a greater complexity. IL-1 signaling was found to control viral replication to some degree (Fig. 2d), but appeared to exert a slight pathological effect, with mice lacking IL-1 signaling more likely to survive infection than those with an intact pathway, despite higher viral loads (Fig. 2c). We have shown that IL-18 is protective, improving both survival and control of viral replication (Fig. 2a, b). IL-18 signaling increased concentrations of interferon gamma in the serum (Fig. 5), although by 7 days after infection, when viral titers are significantly higher in IL-18R^{-/-} mice, IL-18-independent sources of interferon gamma may compensate for the loss of IL-18, and elevated levels of interferon gamma are observed in IL-18R^{-/-} compared to WT. We have shown that certain T cell subsets have decreased production of interferon gamma in IL-18R^{-/-} mice compared to WT mice, and IL-18-dependent interferon gamma production from T cells may account for the reduced cytokine levels in the serum. This interferon gamma defect likely accounts for the poor survival of both Casp-1/11^{-/-} and IL-18R^{-/-} animals.

While Casp-1/11^{-/-} mice have reduced survival compared to WT mice (Fig. 1a), they have better survival compared to IL-18R^{-/-} mice, even following a ten-fold higher viral dose. We believe that this is due to one of two factors; inflammasome-independent processing of IL-18, likely by calpains and other proteases on the surface of cells that process immature IL-18 secreted by dying cells (Sugawara et al. 2001; Omoto et al. 2006; Chung et al. 2009; Omoto et al. 2010) may be protective, or the loss of pathogenic inflammasome substrates in Casp-1/11^{-/-} mice. The former hypothesis is supported by the presence of IL-18 in the serum of some Casp-1/11^{-/-} mice (Fig. 3). However, the levels of inflammasome-independent IL-18 observed are quite low and may not be sufficient to produce any protective effects. Although we have not generated any data that supports the latter hypothesis, there are numerous substrates for the inflammasome, and beyond IL-1, IL-18, and IL-33, they have not been well characterized.

Our finding that IL-1 exerts a minimal effect on survival (Fig. 2c) was surprising, as recent studies with IAV (Ichinohe et al. 2009; Ramos et al. 2012) and WNV (Okamura et al. 1995; Kohno et al. 1997; Bellora et al. 2012; Ramos et al. 2012; Serti et al. 2014) have found that IL-1 signaling is critical to induction of a protective T cell response during infection with these viruses. IL-1 mediates this effect through promotion of DC activation, and mice lacking IL-1 signaling possessed defective DCs and a poorly primed T cell response (Okamura et al. 1995; Dao et al. 1996; Tsutsui et al. 1996; Pang et al. 2013; Durrant et al. 2013). As IL-1R^{-/-} mice actually have slightly improved survival during high-dose MHV infection (Fig. 2c), we do not believe that IL-1 signaling is critical for T cell priming in our model, although the slight elevation in viral replication (Fig. 2d) could be due to a minor T cell defect. The mechanistic

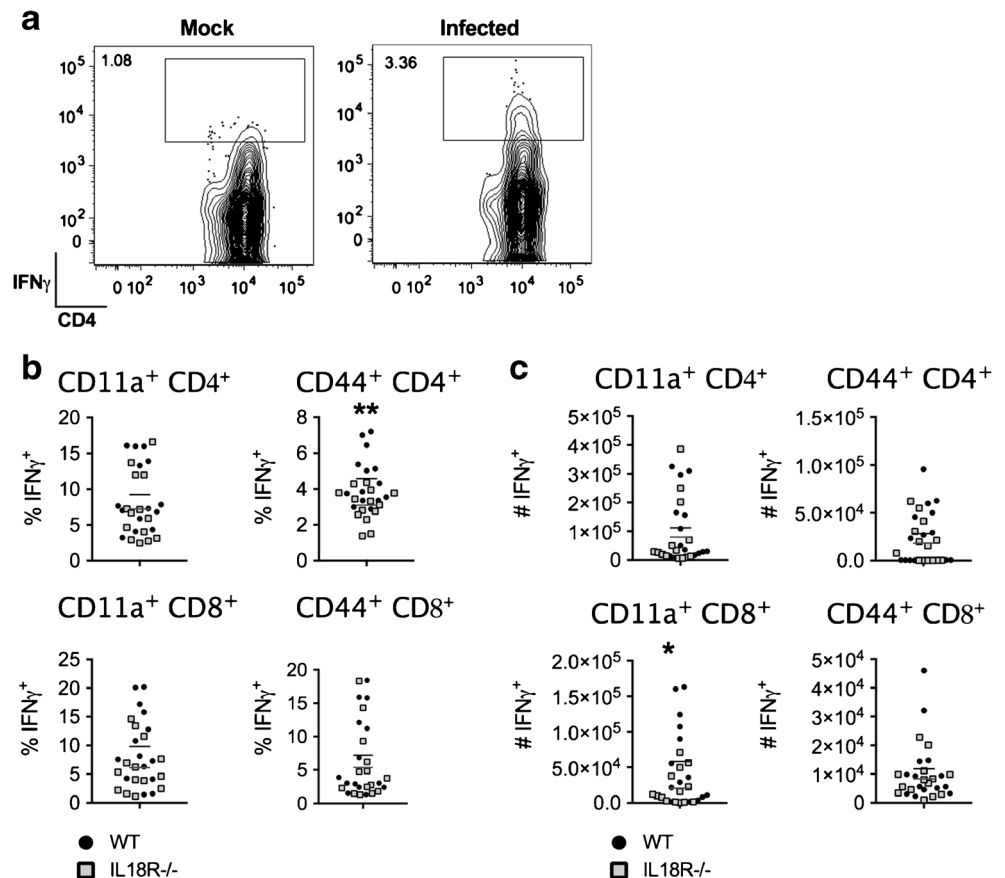
Fig. 6 T cells from WT and IL-18R^{-/-} spleens have similar activation profiles at the peak of the response. WT and IL-18R^{-/-} mice were inoculated i.c. with 500 PFU of MHV. Seven days after infection cells were isolated from the spleen and immunophenotyped by flow cytometry. The percent (a) and number (b) of CD4 and CD8 T cells with elevated expression of the activation markers CD44 or CD11a was determined. Data are pooled from three independent experiments. Statistical significance was determined by an unpaired, two-tailed *t* test



explanation for why IL-1 signaling is critical for DC function during some infections but not others is unknown, and beyond the scope of this work. During IAV infection, IL-1 signaling appears to replace the need for PRR signaling on DCs (Roth-Cross et al. 2008; Pang et al. 2013). It is possible that during MHV infection PRR signaling is sufficient to induce activation, removing the need for IL-1 signaling.

IL-18 has been shown in previous studies to promote cytotoxicity and interferon gamma production in numerous cell types, including T cells and NK cells (Okamura et al. 1995; Dao et al. 1996; Tsutsui et al. 1996; Kohno et al. 1997; Ichinohe et al. 2009; Bellora et al. 2012; Ramos et al. 2012; Serti et al. 2014). We observed a significantly lower concentration of interferon gamma in the serum of IL-18R^{-/-} mice as

Fig. 7 T cells from the spleens of IL-18R^{-/-} mice at the peak of the T cell response exhibit reduced interferon gamma production compared to those from WT mice. WT and IL-18R^{-/-} mice were inoculated i.c. with 500 PFU of MHV. Seven days after infection, cells were isolated from the spleen and immunophenotyped by flow cytometry. Production of interferon gamma was determined by intracellular staining. Representative staining of interferon gamma production by CD4⁺CD44⁺ cells is shown (a). The percent (b) and number (c) of CD11a^{high} or CD44^{high} CD4 and CD8 T cells with elevated interferon gamma production was determined. Data is pooled from three independent experiments. Statistical significance was determined by an unpaired, two-tailed *t* test (**P* < 0.05; ***P* < 0.01)



compared to WT mice 5 days after infection (Fig. 5), which we believe to stem from defective cytokine production from splenic cells (Fig. 6). No defect was observed in T cells isolated from the liver, suggesting that the role of IL-18 may be organ specific. IL-12 signaling might compensate for the loss of IL-18 signaling in the liver. By 7 days after infection, interferon gamma levels were higher in IL-18R^{-/-} mice compared to WT mice, which we attribute to IL-18-independent sources of the cytokine responding to the increased viral replication. However, the decreased interferon gamma levels 5 days after infection, at the peak of interferon gamma production, suggest that interferon gamma production is largely dependent on IL-18 signaling during MHV infection. This may account for the poor survival and control of viral replication of IL-18R^{-/-} mice (Fig. 2a, b). In order to determine if T cells specifically require IL-18 signaling for full interferon gamma production, we isolated cells from the spleen 7 days after infection and assessed their production of interferon gamma by intracellular staining and flow cytometry. We found reduced cytokine production in CD44⁺ CD4 T cells and CD11a⁺ CD8 T cells from the spleen 7 days after infection. Although these defects were detectable only 7 days after infection, when interferon gamma levels were higher in IL-18R^{-/-} mice than WT mice, we speculate that they are indicative of a general defect in the T cell response that leads to the susceptibility of IL-18R^{-/-} mice to MHV infection. The elevated interferon gamma levels in the serum may not be present early enough in infection, or at high enough concentrations at the sites of infection, to compensate for the reduced interferon gamma production by activated T cells, suggesting that IL-18 signaling may play a cell type, organ, and/or temporally specific, but nonetheless critical, role during MHV infection.

Materials and methods

Virus and mice

Recombinant MHV strain A59 (referred to herein as MHV) has been described previously (Phillips et al. 1999; MacNamara et al. 2005; Garlanda et al. 2013). Titer was determined by plaque assay on murine L2 cell monolayers, as described previously (Hingley et al. 1994; Sugawara et al. 2001; Omoto et al. 2006; Omoto et al. 2010). Wild-type C57BL/6 (WT) mice and IL-1 receptor-1 (*Il1r1*)-null mice (Glaccum et al. 1997; Losy and Niezgodna 2001; Karni et al. 2002) (IL-1R^{-/-}) were purchased from Jackson Laboratories (Bar Harbor, ME). IL-18 receptor (*Il18r1* or *il-1rrp*) knockout mice (Hoshino et al. 1999; Shi et al. 2000; Jha et al. 2010; Inoue and Shinohara 2013) (IL-18R^{-/-}) were a generous gift from Dr. Sunny Shin (University of Pennsylvania, Department of Microbiology). Caspase-1 caspase-11 knockout (*Casp1^{-/-}Casp11^{-/-}*) deficient mice (Kuida et al. 1995;

Okamura et al. 1995; Nagarajan and Kronenberg 2007; Bellora et al. 2012; Serti et al. 2014) (*Casp-1/11^{-/-}*) were a generous gift from Dr. Igor Brodsky (University of Pennsylvania, Department of Pathobiology).

All mice were genotyped and bred in the animal facilities of the University of Pennsylvania. Four-to-six-week-old mice were used for all experiments. For infections, virus was diluted in phosphate-buffered saline (PBS) supplemented with 0.75% bovine serum albumin (BSA) and inoculated i.c.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by University of Pennsylvania Institutional Animal Care and Use Committee. All intracranial inoculation was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Viral replication burden

To quantify viral replication, mice were inoculated i.c. with 500 PFU of MHV and sacrificed 3, 5, and 7 days after infection. Following cardiac perfusion with phosphate-buffered saline, organs were harvested and placed in gel saline (an isotonic saline solution containing 0.167% gelatin), weighed, and frozen at -80 °C. Organs were subsequently homogenized, and plaque assays were performed on L2 fibroblast monolayers as previously described (Hingley et al. 1994; Okamura et al. 1995; Dao et al. 1996; Tsutsui et al. 1996).

Serum collection

To isolate serum for ELISAs, blood was incubated for 1 h at 37 °C, then spun at maximum speed in a tabletop centrifuge for 10 min to pellet red blood cells. Serum was removed from the upper phase and frozen at -20 °C until needed.

IL-18 and interferon gamma ELISA

Levels of IL-18 or interferon gamma in serum isolated from infected mice was assayed using capture and detection antibodies specific for IL-18 (MBL) or interferon gamma (BD).

Cell isolation from the spleen and liver

WT and IL-18 receptor deficient (IL-18R^{-/-}) mice were infected i.c. with 500 PFU of MHV, and organs were harvested after CO₂ euthanasia and cardiac perfusion with PBS. Splenocytes were homogenized into single-cell suspensions through a 70-μm filter, after which red blood cells were selectively lysed by incubating for 2 min in 0.206% tris HCl, 0.744% NH₄Cl solution. The livers were homogenized mechanically, and lysates were centrifuged through a Percoll® gradient to obtain a single-cell suspension, followed by RBC lysis as above.

Surface marker and intracellular cytokine staining

Intracellular cytokine staining was performed on single cell suspensions of splenocytes or hepatocytes following a 4-h incubation with brefeldin A (20 µg/ml, Sigma) and the MHV peptides M133 (MHC class II; 4 µg/ml, Biosynthesis) and S598 (MHC class I; 9.3 µg/ml, Biosynthesis). Isolated cells were stained with the following antibodies: CD3 (eBioscience, clone 17A2), CD4 (eBioscience, clone GK1.5), CD8 (eBioscience, clone 53–6.7), CD44 (eBioscience, clone IM7), and CD11a (Biolegend, clone M17/4). Staining for interferon gamma (eBioscience, clone XMG1.2) was performed after permeabilization with Cytotfix/cytoperm Plus Fixation/Permeabilization kit (BD). An LSR II (Becton Dickinson) flow cytometer was used to analyze stained cells. The resulting data were analyzed using FlowJo software (Treestar).

Splenocyte and hepatic cell reculture

Cells were isolated from the spleen and livers of infected mice by the same protocol used prior to flow cytometric analysis. Following isolation, 2×10^6 cells were cultured in wells of a 96-well round bottom plate, in 200-µl media, for roughly 16 h at 37 °C. Supernatants were removed and frozen at –20 °C until analyzed for protein concentration by ELISA.

Statistical tests

Statistical significance was determined by an unpaired, two-tailed *t* test unless otherwise noted. For data sets in which some values were below the limit of detection, significance was instead determined by a two-part testing consisting of a conditional *t* test and proportion test.

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Compliance with ethical standards This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by University of Pennsylvania Institutional Animal Care and Use Committee.

Conflict of interest The authors declare that they have no conflict of interest.

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