Cardiac-Specific Overexpression of Melanoma Differentiation Associated
Gene-5 Protects Mice from Lethal Viral Myocarditis

Philip et al: Cardiac MDA5 and Viral Myocarditis

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Abstract

**Background**—Viral myocarditis is among the most common causes of heart failure in children and young adults. The RNA helicase melanoma differentiation associated gene-5 (MDA-5) is critical for host antiviral responses against members of the *Picornaviridae* family, such as encephalomyocarditis virus (EMCV). MDA5-KO mice are highly susceptible to EMCV infection and develop significant myocardial injury and left ventricular dysfunction. However, the mechanisms by which MDA5 signaling within cardiac myocytes contributes to the host response against viral infection have not been defined.

**Methods and Results**—We generated cardiac-specific MDA5 transgenic (αMH-MDA5) mice. These mice showed increased baseline cardiac expression of antiviral cytokines and increased cellular infiltration but no alterations in cardiac function and/or structure. αMHC-MDA5 mice were less susceptible to EMCV infection and had a significantly lower cardiac viral load compared to littermate (LM) control mice. The severity of myocarditis, prevalence of cardiac myocyte apoptosis and cleavage of caspase 3 after EMCV infection were attenuated in MHC-MDA5 mice. Moreover, MHC-MDA5 mice were protected against EMCV-induced myocardial dysfunction.

**Conclusions**—Our data suggest that myocardial MDA5 may be a key molecule in protecting the heart from direct viral injury and myocardial dysfunction.

**Key Words:** inflammation, infection, innate immunity, cytokines, myocardial function, heart failure
Viral infections of the heart are important causes of morbidity and mortality in children and young adults. Recent evidence suggests that cardiac myocytes possesses a functionally intact innate immune system that becomes activated in response to many forms of acute injury including viral infection. Over the last decade our understanding of the innate immune mechanisms that recognize and respond to viral invasion has greatly expanded. The retinoic-induced gene -I-like receptors (RLRs), including RIG-I, MDA5 (also known as interferon induced with helicase C domain or IFIH1), and LGP2, are a major component of the RNA virus detection system in mammals. In contrast to TLR3 which primarily recognizes dsRNA from within the endosome, RLRs function as cytoplasmic sensors of double stranded RNA. Recent studies have shown that MDA5 is associated with the detection of members of the Picornaviridae family such as EMCV. Consistent with this observation, MDA5-KO mice succumbed after EMCV infection as result of direct viral injury to the heart leading to severe myocardial dysfunction. Similar observations have been reported in the setting of acute CVB3 infection in MDA5-KO mice suggesting that MDA5 signaling may play a critical role in protecting the heart during acute viral infection. Recently we have shown that mice with conditional cardiac-specific overexpression of the TLR3/4 adaptor molecule TRIF, are protected against EMCV-induced mortality, myocarditis and left ventricular dysfunction. An interesting observation that arose from these studies was that the expression of MDA5 was significantly increased in the hearts of TRIF transgenic mice. This raised the intriguing possibility that MDA5 expression could contribute to the protective phenotype observed in the TRIF transgenic mice.

The present study was undertaken to further delineate the role of MDA5 in viral infection of the heart. Accordingly, we generated transgenic mice with cardiac-specific expression of
MDA5 and challenged with a myocarditic variant of the encephalomyocarditis virus (EMCV). Here we show that cardiac-specific overexpression of MDA5 results in the upregulation of antiviral molecules that are essential for efficient control of EMCV; attenuates EMCV-induced myocarditis and cardiac myocyte apoptosis; and prevents EMCV-induced myocardial dysfunction. These findings highlight a crucial role for cardiac MDA5 in the setting of acute viral myocarditis.

Methods

Generation of cardiac specific MDA5 transgenic mice. A transgenic construct containing a 3-kb mouse MDA cDNA was produced by subcloning the c-myc-tagged mouse MDA5 cDNA into the Sal I/Hind III cloning sites downstream of the αMHC promoter (provided by J. Robbins, University of Cincinnati). Southern hybridization was used to detect transgene expression in the heart and c-myc Western blot was used to detect the c-myc-tagged MDA5 protein

MDA5 knock out (MDA5-KO) were provided by Drs. Gitlin and Colonna (Washington University School of Medicine). All experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine and were performed in compliance with the National Institute of Health regulations for animal handling and usage.

Western blot. Protein was separated on 12% SDS-polyacrylamide gel under denaturing conditions. The membrane was immunoblotted with rabbit anti-c-myc and GAPDH (Santa Cruz Biotechnology). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA).
Myocardial cytokine ELISA. Cardiac TNF, CXCL10, IFNγ and IFNβ proteins were measured by ELISA (R&D Systems, Minneapolis, MN). Data were expressed as pg/mg of protein.

Real-time RT-PCR analysis. Pre-developed murine TagMan Gene Expression Assays were purchased from Applied Biosystems: Mx1, IRF7, Isg15, Oas2 and GAPDH. Relative gene expression was calculated by the delta-delta Ct method. Expression of target genes was normalized to GAPDH and reported as fold change vs. the appropriate control.

Echocardiographic analysis. Studies were performed in the Mouse Phenotyping Core at Baylor College of Medicine as previously described.10

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded heart tissue as previously described.10 The pan-leukocyte marker, rat anti-mouse CD45 (BD PharMingen; 1:500) was used to stain infiltrating cells. Biotinylated, HRP-tagged anti-rat antibody (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody at a dilution of 1:200.

Quantification of inflammatory cells. The level of inflammation was determined morphometrically as the volume fraction (% vol) of stained myocardium containing inflammatory cells (CD45+) per high-power field using an unbiased stereological technique as we have previously described.10
**Viral inoculation and titers.** Six-week-old male and female littermate (LM), αMHC-MDA5 and MDA5-KO were inoculated i.p. with 50 plaque-forming units (pfu) of EMCV. Viral titers were determined as we have previously described.11

**Measurement of serum cardiac troponin I levels.** Serum cardiac troponin I (cTnI) levels were measured with a commercially available kit (Life Diagnostics, West Chester, PA).

**Detection of apoptosis.** TUNEL assay was performed using the ApopTaq Plus apoptosis detection kit (Millipore Corp. Billerica, MA). See supplement for details.

**Cleaved caspase 3 western blot.** The membrane was immunoblotted with rabbit anti-cleaved caspase 3 antibody (1:1000; Cell Signaling) and anti-GAPDH antibody (1:5000; Santa Cruz Biotechnology). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1:1000; Santa Cruz). For each sample band density (measured in arbitrary units) was evaluated using ImageJ analysis software

**Statistical analysis.** All values were expressed as mean ± SEM. A Kaplan-Meier log-rank analysis (14 days) was used to compare survival curves between LM and MHC-MDA5 mice. Pair-wise comparisons were performed using Mann-Whitney; comparisons between more than two groups was performed by a one way ANOVA and post-hoc ANOVA testing (Fisher’s PLSD) was performed where appropriate (StatView Adept Scientific Inc., Acton, MA).
Results

Characterization of αMHC-MDA5 Mice

We obtained 2 heterozygous founder lines (Figure 1A) that harbored cardiac restricted overexpression of the MDA5 transgene (αMHC-MDA5): 1995 (~5 copies) and 524 (~25 copies). Both founder lines grew to adulthood without increased mortality. Western blot analysis of cardiac extracts from both founder lines confirmed increased myocardial expression of the c-myc-tagged MDA5 protein (Figure 1B). The αMHC-MDA5-1995 line was selected for further characterization and is referred as αMHC-MDA5.

To determine whether cardiac specific overexpression of MDA5 led to increased expression of cytokines in the heart, we performed ELISA to measure TNF, CXCL10, IFNβ and IFNγ expression. As shown in Figure 1C, TNF, IFNβ and IFNγ protein expression was significantly (P < 0.05) increased in the hearts of αMHC-MDA5 mice when compared with LM mice. In contrast, there were no differences in cardiac CXCL10 protein expression between the 2 groups of mice. Because overexpression on MDA5 led to production type I interferon in the heart, we assessed whether this also led to expression on interferon-stimulated genes (ISGs). Figure 1D shows that αMHC-MDA5 mice had increased cardiac mRNA expression of Irf7, Mx-1, Isg15 and Oas2 when compared to LM mice.

Phenotypic Characterization of αMHC-MDA5 Mice (6 weeks)

Figure 2 summarizes the baseline assessment of cardiac structure and function in six-week old LM and αMHC-MDA5 mice. The heart-weight to body-weight ratio was not significantly different in the 2 groups of mice (Fig. 2A). Similarly, cardiac function as measured by ejection
fraction (% EF; Figure 2B and C) did not differ between the 2 groups. Cardiac troponin I was not detected in the sera of LM or αMHC-MDA5 mice at six weeks of age (data not shown).

Heart sections were stained to assess for baseline pathological alterations in LM and αMHC-MDA5 mice. The overexpression of MDA5 resulted in spontaneous cellular infiltrates in the myocardium of αMHC-MDA5 mice (Figure 2D). The percentage of infiltrating CD45+ cells (Figure 2E) was significantly greater in the hearts of αMHC-MDA5 (2.6 ± 0.4%) compared to LM controls (0.7 ± 0.15%; P <0.05). Despite increased myocardial expression of TNF, ISGs and spontaneous white cell infiltration, no obvious cardiomyocyte disarray, necrosis or cardiac dysfunction were noted in αMHC-MDA5 mice.

αMHC-MDA5 mice exhibit decreased susceptibility to EMCV-induced mortality
To determine whether cardiac specific overexpression of MDA5 affected the susceptibility to virus-mediated lethality and cardiac injury, we challenged LM (n=25), αMHC-MDA5 (n=25) and MDA5-KO mice (n=25) with a myocarditic variant of EMCV. EMCV-infected -MDA5-KO mice had significantly earlier mortality when compared with αMHC-MDA5 transgenic mice and their LM controls (Figure 3). By day 4 after infection, 100% of MDA5-KO mice were dead. In contrast, 100% of αMHC-MDA5 and 60% of LM were alive at day 4 after infection. By day 14 after infection 15% of LM and 46% of αMHC-MDA5 mice were alive. Kaplan Meier analysis showed a significant (P<0.05) difference in the 14-day survival between LM and αMHC-MDA5 mice.
Cardiac Specific αMDA5 Expression Attenuates Severity of Myocarditis

To determine whether MDA5 overexpression also affected the severity of myocarditis in EMCV-infected mice, quantitation of CD45+ cells was performed at 3, 5 and 14 days after infection. Figures 4A and B show an increase (P <0.05) in the percentage of CD45+ cells in the hearts of both LM and αMHC-MDA5 mice at 3, 5 and 14 days post infection. However, the degree of myocardial inflammation was significantly greater in EMCV-infected LM mice. When baseline differences in cardiac CD45+ cells were taken into consideration, the fold change in CD45+ cells was significantly greater in LM mice (data not shown). We performed immunohistochimistry for CD4, CD8, CD206 (M2), and CD11c (M1) and well as quantitative PCR on hearts from both groups. Prior to EMCV infection there was a paucity of all cell types in the hearts of LM mice (Suppl. Figure 1A). At baseline the cellular infiltrate in the hearts of αMHC-MDA5 mice was primarily composed of CD11c (M1 phenotype) and CD8+ T cells. These findings were also supported by the qPCR data (Suppl. Figure 1B). We also noted marked staining for CD206 (M2 phenotype) macrophages in the hearts of αMHC-MDA5 mice despite a very modest increase in CD206 mRNA expression. After infection, there was a dramatic increase in CD8+ T cells and CD11c (M1 phenotype) and in the hearts of LM mice (Suppl. Figure 2A). In contrast, these increases were dampened in the hearts of αMHC-MDA5 mice. Interestingly, we noted decreased staining of CD206+ cells (M2 phenotype) in the hearts of LM mice at day 3 after infection compared to αMHC-MDA5 mice. A similar trend was noted for CD206 mRNA expression (Suppl. Figure 2B).

Figure 5 shows that cardiac protein levels of CXCL10 and IFNβ were significantly increased in the hearts of LM mice at day 3 and 5 after infection. In contrast, the TNF protein levels were significantly higher in the hearts of infected αMHC-MDA5 mice. In addition, there
was a trend towards higher INFγ levels at days 3 and 5 after infection in the hearts of αMHC-MDA5 mice. Figure 5B also shows that cardiac mRNA expression of Ifn7, Mx-1, Isg15 and Oas2 increased in both LM and αMHC-MDA5 mice following infection. However, the changes were significantly greater in the hearts LM mice. These data suggest that increased baseline expression of antiviral mediators modulated the response mounted by the infected host cell in αMHC-MDA5 mice.

**Decreased virus replication and myocardial injury in αMDA5 transgenic mice.**

To assess the biological significance of cardiac restricted MDA5 overexpression we measured cardiac viral load after infection. Figure 6A shows that LM and MDA5-KO mice had significantly (P<0.5) higher viral loads in the heart at 3 days after infection when compared to αMHC-MDA5 mice. EMCV titers were 100-fold and 1000-fold higher in the hearts of EMCV infected LM and MDA5-D mice at 3 days, respectively. By day 5 post infection viral replication was decreasing in the hearts of both LM and αMHC-MDA5 mice, but they were still significantly higher in LM mice (Figure 6A). EMCV titers in the liver were not significantly different between LM and αMHC-MDA5 mice at day 3 (3.4± 0.05 vs. 3.2 ±0.05 PFU log10/mg tissue). In contrast, on day 3 after EMCV infection viral load in the livers of MDA5-KO (4.2± 0.30 vs. 3.2 ±0.05 PFU log10/mg tissue) were 10-fold higher than in LM or αMHC-MDA5 livers.

To determine whether decreased mortality in the αMHC-MDA5 mice was associated with decreased myocardial injury, we measured serum cTnI levels on days 3 and 5 after infection. We found that on days 3 and 5 post infection, serum cTnI levels were significantly lower in αMHC-MDA5 mice as compared with that in LM mice (Figure 6B). This suggested that cardiac overexpression of MDA5 attenuated viral induced cell lysis.
To determine whether MDA5 overexpression in the cardiac myocyte is sufficient to affect cardiac function following EMCV infection, echocardiography was performed before and at 4 and 7 days after infection. LV function was normal in both LM and αMHC-MDA5 mice before infection. At 4 days after EMCV infection, LV function and end systolic dimensions were near normal in both groups (Figure 6C). By day 7 after infection there was a significant decrease in the ejection fraction and an increase in chamber dilation in LM mice. The latter was manifested as a significant increase in LVESD (Figure 6C and D). Thus, cardiac myocyte-specific overexpression of MDA5 resulted in a significant decrease in virus replication and myocardial injury, leading to preservation of left ventricular function. These data suggest that MDA5 signaling within the cardiac myocyte is an important innate antiviral defense mechanism.

**EMCV-induced myocyte apoptosis is attenuated in αMDA5 transgenic mice.**

MDA5 is has been shown to initiate a proapoptotic signaling pathway that is independent of type I IFNs. From this point of view, the MDA5-mediated induction of apoptotic signaling during viral infection could represent a defense strategy of the host that counteracts the anti-apoptotic activities of the invading pathogen. To determine whether cardiac specific overexpression of MDA5 was sufficient to induce apoptosis, we compared the prevalence of apoptotic cardiac nuclei in 6-weeks old αMHC-MDA and LM control mice. Figures 7A and B show that the prevalence of cardiomyocyte apoptosis was higher (0.0125 vs. 0.025; P< 0.05) at baseline (BL) in αMHC-MDA5 than in LM mice. Importantly, these differences in BL apoptosis did not lead to changes in myocardial structure or function (Figure 2). Following infection (days 3 and 5), the prevalence of cardiomyocyte apoptosis increased significantly in both groups. However, the increase in apoptotic nuclei was more dramatic in LM infected mice (Figures 7A and B). We
also assessed caspase 3 cleavage by western blot as another measure of apoptosis. No significant differences in the BL levels of cleaved caspase 3 (Figures 7C and D) were noted between LM and αMHC-MDA5 mice. EMCV infection (days 3 and 5) induced a significant increase in the levels of cleaved caspase 3 in the hearts of both LM and αMHC-MDA5 mice. In agreement with the TUNEL staining data, the changes in cleaved caspase 3 were more pronounced in the hearts of EMCV-infected LM mice.

**Cardiac specific overexpression of αMDA5 prolongs survival in MDA5 deficient mice.**

To further define the role of cardiac MDA5 expression MDA5-KO mice were interbred with animals overexpressing a MDA5 transgene (αMHC-MDA5) exclusively in the heart. MDA5-KO mice develop severe myocarditis and experience 100% early lethality (day 3-4). Survival of after EMCV infection was compared between MDA5-KO and αMHC-MDA5/MDA5-KO double mutant mice. Figure 8A depicts the survival curves of the two strains of animals. Cardiac expression of MDA5 markedly enhanced the 14-day survival of MDA5-KO mice. That is, survival improved in MDA5-KO mice from 0% to 21% and was similar to the survival of EMCV-infected LM mice (15%; Figure 3). Furthermore, cardiac viral load in αMHC-MDA5/MDA5-KO double mutant mice were 10 times lower compared to MDA5-KO mice (Figure 8B). Figure 8C shows that there was a trend toward lower cardiac troponin levels in αMHC-MDA5/MDA5-KO double mutant mice.

**Discussion**

Viral infections of the heart are an important cause of heart failure. Although the discovery and characterization of RLRs has led to a better understanding of the innate immune system, the
mechanisms that contribute to host defense against viral infection in the cardiac myocyte are only partially understood. Recent studies have shown that global loss of function of either MDA5 or its adaptor molecule MAVS increases the susceptibility of adult mice to EMCV and CBV3-induced mortality and multi-organ failure (pancreas, liver and heart). However, the aforementioned studies with total knockout mice cannot be used as evidence to support the importance of cardiac MDA5 signaling in the pathogenesis of viral myocarditis.

Here we have taken a genetic approach to examine the role on MDA5, within cardiomyocytes, in the development of acute EMCV-induced myocarditis. Our characterization of αMHC-MDA5 mice shows that overexpression of MDA5 in cardiomyocytes can lead to spontaneous baseline expression of cardiac cytokines including TNF, INFα, IFNγ, and ISGs. Baseline expression of these mediators was associated with mild cardiac inflammation but no evidence of cardiac dysfunction (Figure 1). The results from the present study show for the first time that cardiac-specific overexpression of MDA5 attenuates EMCV-induced mortality, cardiac viral replication, myocarditis, and cardiac myocyte apoptosis; and results in the prevention of left ventricular dysfunction and cardiac dilation following infection. Moreover, cardiac targeted expression of MDA5 improved 14 day survival by 21% in MDA5-KO mice. Using a similar transgenic approach we have previously shown that cardiac-specific overexpression of the TLR3/TLR4 adaptor molecule TRIF, protected cardiac myocytes from direct EMCV injury and attenuated cardiac dysfunction. Interestingly, cardiac-specific TRIF transgenic mice exhibited increased baseline expression of the RLRs MDA5 and LGP2 suggesting that these molecules may synergize to control EMCV replication in the heart. In support of this point of view, recent studies have shown that both dengue virus and rhinovirus require co-triggering of TLR3/TRIF and MDA5 to induce an adequate innate immune response to control viral replication.
Although the mechanisms for the resistance to EMCV infection in αMHC-MDA5 mice have yet to be completely defined, the observed increased baseline expression of TNF, INFγ, INFβ, and ISG may explain this effect as EMCV replication has been shown to be inhibited by their antiviral effects. The exogenous administration of TNF or INFβ has been shown to modify the severity of EMCV or CVB3-induced myocarditis in mice.\textsuperscript{15-17} However, the protective effects in the heart following administration of a single agent do not appear to be complete. Wessely et al\textsuperscript{18} reported that type I IFN signaling played an essential role in preventing early CVB3 replication in the liver but had little effect on early viral replication in the heart. These findings questioned the role of endogenous type I interferon signaling in the control of viral replication in the heart. However, Riad et al\textsuperscript{15} have recently shown that exogenous administration of IFN-β improves survival and decreases CVB3 replication in TRIF knockout mice. Although the role of IFNβ in the treatment of patients with acute viral myocarditis remains unclear, a recent study has suggested that IFNβ may be beneficial in the treatment of chronic viral cardiomyopathy.\textsuperscript{19} In the present study, MDA5 overexpression had a marked effect on cardiac viral replication and cardiac injury, suggesting that the early presence of multiple cytokines such as TNF, IFN-β, IFN-γ, and ISGs may be required to effectively protect the cardiac myocyte against direct viral injury. The factors that may affect the expression levels of MDA5 in the heart are unknown. While there have no studies describing any associations between MDA5 mutations and host susceptibility to viral myocarditis, there have been reports linking mutations in \textit{IFIH1} (MDA5) to type I diabetes.\textsuperscript{20-22} Resistance to type I diabetes is associated with polymorphisms that reduce MDA5 protein expression and impair the function of MDA5. Whether the presence of these SNPs will have any effect on the pathogenesis of viral heart disease is unknown.
The induction of apoptosis is an innate immune mechanism by which host cells protect themselves from lytic viruses such as EMCV. Signaling through MDA5 and its adaptor molecule MAVS has been shown to induce apoptosis in a number of cells. Interestingly, picornaviruses viruses have evolved strategies to manipulate the cellular apoptotic machinery with the goal of prolonging the survival of the infected cell, which in turn helps the virus to complete its life cycle. From this point of view, the MDA5-mediated induction of apoptotic signaling reflects a defense strategy of the host that counteracts the anti-apoptotic activities of the invading pathogen. However, experimental and clinical studies have shown that virus-induced cardiomyocyte apoptosis also has a deleterious effect on left ventricular function leading to severe heat failure and in severe cases death. In the present study, EMCV-induced cardiac myocyte apoptosis and caspase-3 cleavage were attenuated in αMHC-MDA5 transgenic mice when compared to infected LM controls. The decreased prevalence of apoptosis and caspase-3 cleavage in αMHC-MDA5 mice can be explained, at least in part, by the significantly lower viral loads in the hearts of these mice. From a pathophysiological standpoint, the left ventricular dysfunction observed in LM infected mice is likely the result of ongoing viral replication and progressive cardiomyocyte apoptosis.

The results from this study have highlighted the beneficial effects of MDA5 signaling in the adult mammalian heart. However, following resolution of the acute infection MDA5 activation may represent a mixed blessing for the heart. That is, the controlled self-limited activation of this pathway is critical in activating host defense mechanisms (TNF, INFβ and IFNγ) in the heart, but uncontrolled and/or prolonged activation of MDA5 signaling may produce devastating consequences leading to persistent inflammation, myocardial dysfunction, dilated cardiomyoapthy and death. For example, mice with targeted overexpression of TNF in
the cardiac compartment develop florid myocarditis, progressive myocardial fibrosis and heart failure.\textsuperscript{32-34} Similarly, IFNγ transgenic mice also develop dilated cardiomyopathy and a reduction in fractional shortening that is mediated through increased production of TNF.\textsuperscript{35} Interestingly, Cramptom et al\textsuperscript{36} recently reported that MDA5 transgenic mice have increased levels of type I interferon and ISGs, are resistant to infection with vesicular stomatitis virus, but do not spontaneously develop autoimmune or inflammatory pathology. These results are in agreement with what we have reported here except that αMHC-MDA5 mice do show spontaneous production of cardiac inflammatory cytokines (TNF and IL1), but do not develop myocardial dysfunction at six-weeks of age.

In conclusion, targeted expression of MDA5 in the cardiac myocyte prevents EMCV-induced contractile dysfunction, is associated with decreased cardiac viral replication, less severe myocarditis, and a decreased prevalence of cardiac myocyte apoptosis. Interestingly, we observed a trend toward an M2 macrophage phenotype which could potentially promote repair while limiting secondary inflammatory-mediated injury in αMHC-MDA5 mice. Furthermore, the significant improvement in survival in αMHC-MDA5/MDA5-KO mice compared with MDA5-KO mice provides further evidence of the importance of MDA5 signaling in the heart in the setting of acute viral myocarditis. More importantly is the fact that the protective effects were noted despite continued viral replication in the livers of the αMHC-MDA5/MDA5-KO mice. The important question that arises from this study is whether it will be possible to modulate the potential maladaptive consequences of MDA5 activation and proinflammatory cytokine expression while preserving the beneficial antiviral effects. It seems plausible that even after resolution of the infection the persistent activation of the MDA5 pathway may lead to
pathological consequences. The answer to this important question will come from the longitudinal observation of αMHC-MDA5 mice.

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**Disclosures**

None.

**References**


Figure Legends

Figure 1. Generation of MDA5 transgenic mice. (A) MDA5 gene integration was confirmed by Southern Blot analysis. (B) Western blot analysis confirmed MDA5 myc-tagged protein overexpression in cardiac tissue. (C) Baseline (BL) cardiac cytokine expression in heart of LM and αMHC-MDA5 mice at 6 weeks of age. (D) BL cardiac mRNA expression of Mx1, Irf7, Isg14 and Oas2. The values from LM mice were set to 1, and the relative mRNA units represent the fold induction over LM mice (n= 5 mice per group). mRNA expression was normalized to Gapdh. All data are presented as means ± SE (*P<0.05).

Figure 2. Phenotypic characterization of αMHC-MDA5 transgenic mice. (A) Baseline heart weight: body weight ratios and (B) LV ejection fraction (EF) in LM and αMHC-MDA5 mice at 6 weeks of age (n=5 per group). (C) M-mode tracings. (D) BL cellular infiltration in hearts of LM and αMHC-MDA5 mice at 6 weeks of age (n=7-10 hearts per group). (E) Quantitation of CD45+ cells in the myocardium of LM and αMHC-MDA5 mice.

Figure 3. Cardiac specific overexpression of MDA5 increases survival after EMCV infection. LM, MDA5 deficient and αMHC-MDA5 transgenic mice were infected with 50 PFU of EMCV. αMHC-MDA5 mice had significantly increased survival at 14 days. P values obtained using a Log rank test.
*P < 0.05. LM controls vs. αMHC-MDA5 mice and MDA5KO vs. αMHC-MDA5 mice

Figure 4. Histological analysis of EMCV-infected heart tissues. (A) Representative CD45+ stained sections of hearts from LM and αMHC-MDA5 transgenic mice at baseline (BL) and days 3, 5 and 14 after infection (40x). (B) Quantitation of CD45+ cells in the myocardium. Data are presented as means±SE (*P<0.05).

Figure 5. Effect of EMCV infection on cardiac cytokine and chemokine expression. (A) TNF, CXCL10, IFNβ and IFNγ protein levels in the hearts of LM and αMHC-MDA5 mice at day 3 and 5 after EMCV infection. (B) ISG mRNA expression in the heart at BL and days 3 and 5 after infection. (B) Data are presented as means±SE (*P<0.05).

Figure 6. MDA5 overexpression inhibits EMCV replication and attenuates cardiac dysfunction. (A) Viral titers in hearts at 3 and 5 days after infection. (B) Cardiac troponin I levels in EMCV infected mice. (C) Echocardiographic analysis of cardiac function (% EF) and LVESD in LM and αMHC-MDA5 mice at BL and days 4 and 7 after EMCV infection. (D) M-mode tracings. Data are presented as means±SE (*P<0.05 LM vs. αMHC-MDA5; #P < 0.05 MDA5-KO vs. LM and αMHC-MDA5 at day 3.
Figure 7. Cardiomyocyte apoptosis. (A) The prevalence of cardiomyocyte apoptosis was determined by TUNEL staining LM and αMHC-MDA5 at BL and at days 3 and 5 after EMCV infection. (B) Group data are presented as means±SE (*P<0.05). (C) Caspase 3 cleavage after EMCV infection. (D) Group data are presented as means±SE (*P<0.05).

Figure 8. Targeted cardiac expression of MDA5 improves survival in EMCV-infected MDA5 deficient mice (A; P values obtained using a Log rank test.). (B) EMCV replication in the heart of αMHC-MDA5, αMHC-MDA5/MDA5-KO and MDA5-KO Data are presented as means±SE; *P<0.05 αMHC-MDA5 vs. LM and MHC-MDA5/MDA5-KO mice; # P<0.05 MHC-MDA5/MDA5-KO vs. MDA5-KO mice (one-way ANOVA; Fisher’s PLSD).
Figure 1

A

Founder Line

50 25 10 5 1 LM 1 2

mda-5 gene copy #

B

LM | αMHC-MDA5
1 2 | 1995 524

c-myc

tubulin

c-myc Expression Fold-increase vs. LM

LM | F1 | F2

C

TNF

CXCL10

D

INFB | INFγ

Mx1 | Irf 7 | Isg15 | Oas2

Fold-increase vs. LM

LM (n=8) αMHC-MDA5 (n=8)

Circulation
Heart Failure

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American Heart Association

0 2 4 6 8 10

0 2 4 6 8

0 2 4 6 8

0 2 4 6 8
Figure 2

A) HW/BW Ratio

B) Ejection Fraction (%)

C) Waveforms showing differences in heart function between LM and αMHC-MDA5.

D) Histological images of LM and αMHC-MDA5 tissues.

E) Graph showing %CD45 Cells BL comparison between LM and αMHC-MDA5 with n=5.
Figure 3

- LM (n=25)
- αMHC-MDA5 (n=25)
- MDA5-KO (n=25)

% Survival vs. Days after infection
Figure 4

A

LM (n=9)
D
MHC-MDA5 (n=9)

Days post infection

%CD45 Cells

BL D3 D5 D14

B

Circulation
Heart Failure

LM (n=9)
αMHC-MDA5 (n=9)

%CD45 Cells

BL D3 D5 D14

Days post infection
Figure 6

A

EMCV (PFU Log_{10} mg tissue)

LM (n=10)
αMHC-MDA5 (n=10)
MDA5-KO (n=7)

D3
D5

B

cTn-I (ng/ml)

LM (n=6)
αMHC-MDA5 (n=6)

C

Ejection Fraction (%)

LM (n=10)
αMHC-MDA5 (n=10)

BL
D3
D7

D

LVESD (mm)

LM
αMHC-MDA5

BL
D4
D7

ND
Figure 7

A

BL

D3

D5

LM

αMHC-MDA5

B

Prevalence of Apoptosis (%)

LM (n=9)

αMHC-MDA5 (n=9)

C

BL

D3

D5

LM

αMHC-MDA5

Caspase 3

GAPDH

D

Fold-increase vs. BL

Caspase 3