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ROLE OF THE INNATE IMMUNE SYSTEM IN ACUTE VIRAL MYOCARDITIS

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Abstract

Although the adaptive immune system is thought to play an important role in the pathogenesis of viral myocarditis, the role of the innate immune system has not been well defined. To address this deficiency, we employed a unique line of mice that harbor a genomic "knock in" of a mutated TNF gene lacking the AU rich element (TNF^{ARE/ARE}) that is critical for TNF mRNA stability and translation, in order to examine the contribution of the innate immune system in encephalomyocarditis-induced myocarditis (EMCV). Heterozygous mice (TNF^{ARE/+}) were infected with 500 plaque-forming units of EMCV. TNF^{ARE/+} mice had a significantly higher 14-day mortality and myocardial inflammation when compared to littermate control mice. Virologic studies showed that the viral load at 14 days was significantly lower in the hearts of TNF^{ARE/+} mice. TNF^{ARE/+} mice had an exaggerated proinflammatory cytokine and chemokine response in the heart following EMCV infection. Modulation of the innate immune response in TNF^{ARE/+} mice by the late administration of prednisolone resulted in a significant improvement in survival and decreased cardiac inflammation, whereas early administration of prednisolone resulted in a blunted innate response and increased mortality in littermate control mice. Viewed together, these data suggest that the duration and degree of activation of the innate immune system plays a critical role in determining host outcomes in experimental viral myocarditis.

Keywords

myocardial inflammation; innate immunity; cytokines

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INTRODUCTION

Viral infection of the myocardium produces myocardial necrosis and an inflammatory cellular infiltration that often culminates in acute or chronic heart failure [8]. Experimental models of viral myocarditis suggest that the acute phase of the disease process involves both a direct viral cytopathic effect and activation of the host cellular immune response [12,18]. Although the cellular immune response may attenuate viral replication and protect the heart, it also can contribute to the development of myocardial inflammation and necrosis. In deed, depletion of T lymphocytes attenuates myocarditis and mice deficient in both CD4⁺ and CD8⁺ cells or the T-cell receptor β have improved survival and decreased cardiac injury following challenge with coxsackievirus B3 [23]. More recently it has been shown that the T cell receptor-associated tyrosine kinase, p56 *lck*, is critical for both virus replication in the heart and activation of T-cells to target the heart [17]. p56 *lck* deficient mice do not develop myocarditis when challenged with coxsackievirus. Despite the wealth of studies that have shown an important role for the cellular immune response in viral myocarditis, surprisingly little is known about the contribution of innate immune mechanisms to the pathogenesis of viral heart disease.

The basic function of the innate immune system is the restriction of early proliferation of infectious agents. Numerous molecules and effector cells work in concert to restrict the initial spread of an infectious focus. Several lines of evidence suggest that mediators of the innate immune system, such as tumor necrosis factor (TNF) and nitric oxide (NO), play an important role in the pathogenesis of viral myocarditis as well as other cardiomyopathies [10,21,29,32]. For example, elevated levels of TNF have been reported in patients with viral myocarditis and TNF mRNA and protein are consistently upregulated in the hearts of these patients [22;24]. In animal models, the exogenous administration of TNF aggravates myocarditis, and the neutralization of TNF by antibodies or soluble receptors attenuates the disease [16,31]. However, Wada et al.[28] recently reported that mice with a targeted disruption of the TNF gene (TNF^{-/-}) have increased mortality after infection with encephalomyocarditis virus compared with wild-type mice (TNF^{+/+}). Furthermore, exogenous administration of TNF prevented the early increase in virus-induced mortality in the TNF^{-/-} mice. Viewed together, these observations suggest that innate immune mechanisms may play a dual role in the setting of viral myocarditis. Accordingly, in order to better define the role of the innate immune system in viral myocarditis, in the present study we have used a unique line of mice that harbor a genomic “knock in” of a mutated TNF gene lacking the AU rich element that is critical for TNF mRNA stability and translation (TNF^{ARE/-}) [15]. Because heterozygous mice (TNF^{ARE/+}) have normal baseline TNF levels and exaggerated TNF levels following systemic infection, they provide a unique experimental model system in which to explore the role of the innate immune system in viral myocarditis.

MATERIAL and METHODS

Experimental Model

For these studies we used heterozygous 6-8 week old male and female TNF^{ARE/+} mice that harbor a genomic “knock in” of a mutated TNF gene lacking the AU rich element. The AU

rich element is a critical *cis*-acting regulatory motif in the 3' untranslated region (3'UTR) that binds *trans*-acting proteins, which in turn modulate TNF mRNA stability and translation [15]. Age and sex-matched wild-type littermates (TNF^{+/+}) were used as the appropriate controls. TNF^{ARE/+} heterozygous mice have normal basal TNF expression, but have exaggerated TNF levels following simulation with lipopolysaccharide because of increased mRNA stability [15]. Mice were maintained in microisolator cages and were fed pellet food and water ad libitum. All experiments were approved by 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 (NIH publication No. 86-23, revised 1995).

Survival in EMCV infected TNF^{ARE/+} mice

Experimental myocarditis was induced by inoculating (i.p.) male and female littermate control and TNF^{ARE/+} mice with 500 plaque-forming units (pfu) of a myocarditic variant of the encephalomyocarditis virus (EMCV; a generous gift from Dr. Sally Huber). The viral stock was stored at -80°C in RPMI 1640 medium (Invitrogen, Carlsbad, CA) until use. For the purpose of this study the day of virus inoculation was defined as "day 0." Mice were monitored from day 0 to day 14, and the number of animals that were alive was enumerated daily.

Myocardial inflammation in EMCV infected TNF^{ARE/+} mice

Hearts from EMCV infected littermate control and TNF^{ARE/+} mice were fixed on day 7 and 14 via retrograde perfusion of the aorta, using 10% buffered formalin. Transverse myocardial sections were stained with hematoxylin and eosin, and observed by light microscopy at 40x magnification to determine the degree of cellular infiltration. All sections were examined in a blinded manner by an independent investigator, who examined 16 fields per heart. The degree of myocardial inflammation was scored semi-quantitatively in the following manner: 0 = no infiltrate; 1+ = infiltrates involving <25% myocardium; 2+ = infiltrates involving 25% to 50%; 3+ = infiltrates involving 50% to 75% of the myocardium; and 4+ = infiltrates involving 75% to 100% of the myocardium, as described by Kanda et al [13].

Viral replication in TNF^{ARE/+} mice

Myocardial EMCV replication in the littermate control and TNF^{ARE/+} mice was determined by reverse transcription-polymerase chain reaction (RT-PCR) of EMCV mRNA as well as by measuring EMCV viral titers from the hearts of infected mice.

RT-PCR—Mice were sacrificed with a lethal injection of sodium pentobarbital (80 mg/kg) seven days after EMCV inoculation, their hearts harvested, and total RNA extracted using the guanidinium thiocyanate/phenol method, as previously described [2]. RT-PCR was performed using a commercially available kit (Roche Diagnostics, Indianapolis, IN). The following oligonucleotide primer pairs were synthesized: EMCV sense, 5'-GTCGTG-AAGGAAGCAGTTCC-3'; antisense, 5'-CACGTGGCTTTTGGC-CGACAGAGGC-3'; β-actin sense, 5'-GGACTCCTATGTGGGTGA-CGAGG-3'; antisense, 5'-GGGAGAGCATAGCCCTCGTAGAT-3' [28]. The PCR products were analyzed by

agarose gel electrophoresis with ethidium bromide staining. The optimum number of cycles was determined experimentally for each gene product, as well as to verify uniform amplification.

Viral titers—The titer of infectious EMCV was determined in individual heart homogenates by plaque assay, using HeLa cells as described previously [11]. Briefly, heart samples were homogenized in 800 μ L RPMI 1640 and supernatants were stored at -80°C until used in the plaque assay. Dilutions of tissue supernatants were incubated on 80% confluent HeLa cell monolayers for 2 hour at 37°C and 5% CO_2 to allow viral attachment, and then incubated for 2 days to allow plaque formation. All viral titers are expressed as the mean $\pm$ SEM $\log_{10}$ pfu/mg myocardial tissue.

Myocardial proinflammatory cytokine and chemokine expression in TNF^{ARE/+} mice

Ribonuclease protection assay (RPA)—Mice were sacrificed on day 0, 3, 5 and 7 following EMCV infection and total RNA was extracted from the hearts, as described previously [2]. The level of gene expression for tumor necrosis factor (TNF), interleukin 1-beta (IL-1 β), monocyte chemoattractant protein-1 (MCP-1), regulated on activation normal T-expressed and secreted chemokine (RANTES), interferon inducible protein 10 (IP-10), interferon- β (IFN- β) and interferon- γ (IFN- γ) was determined with a multi-probe RNase protection assay system, exactly according to the manufacturer's suggestions (RiboQuant, Pharmingen, San Diego, CA). Signals were quantified using Image QuaNT software and normalized to L32 (Molecular Dynamics, Sunnyvale, CA).

Myocardial TNF and IL-1 β protein levels—Hearts were harvested on day 7 following EMCV infection, and myocardial homogenates prepared as previously described [2]. Myocardial TNF and IL-1 β protein levels were measured by ELISA (R&D Systems, Minneapolis, MN). Data are expressed as pg/mg myocardial protein.

Role of innate and adaptive immunity in EMCV myocarditis

Early and late administration of prednisolone—To delineate the respective roles of the early innate and late adaptive immune responses following EMCV myocarditis, we treated EMCV infected littermate control and TNF^{ARE/+} mice with daily subcutaneous injections of prednisolone (10mg/kg) from day 1-7 (early), in order to attenuate the innate immune response, or with daily subcutaneous injections of 10mg/kg prednisolone from day 7-14 (late), as described by Tomioka et al. [27] For these latter studies we monitored the 14 day survival, myocardial histopathology (day 14), EMCV viral titers (day 7 and 14) and levels of myocardial TNF protein (day 7), as described above.

Neutralizing antibody assay—To determine whether administration of prednisolone affected the circulating levels of neutralizing EMCV antibody titers, we collected blood (sterile manner) from the prednisolone treated littermate control and TNF^{ARE/+} mice 7 and 14 days after EMCV infection at time of sacrifice. Sera were heat inactivated (56°C for 30 minutes) prior to performing serial dilutions. Serial dilutions (1:2) of heat-inactivated serum were prepared in RPMI 1640 medium (Invitrogen) containing 2% fetal bovine serum. To each serum dilution a 100 TCID₅₀ EMCV was added and incubated for 90 minutes at 37°C .

The mixture was then added to HeLa cells cultured in 96-well plates and incubated at 37 °C (95% O₂, 5% CO₂) for 96 hours. Antibody titers were expressed as log₂ of the reciprocal of the last dilution of serum that neutralized 50% of the viral inoculum. The negative control was uninfected mouse serum.

Statistical analysis

All values were expressed as mean ± SEM. The survival rates of mice were analyzed by the Kaplan-Meier method. A two-way analysis of variance was used to test for differences in EMCV-induced proinflammatory cytokine and chemokine expression, as well as viral load. Where appropriate, post-hoc testing was performed using Fisher's protected least-squares difference (PLSD) test to detect mean differences between groups. Cardiac histopathological scores were examined by the Mann-Whitney test. Significant differences were said to exist at $p < 0.05$.

RESULTS

Survival in EMCV infected TNF^{ARE/+} mice

To determine whether sustained activation of the innate immune system affects the host response to viral infection, we inoculated TNF^{ARE/+} mice ($n = 24$) and their wild-type littermate controls ($n = 24$) with EMCV. The Kaplan Meier curves depicted in Figure 1 show that survival for the EMCV infected wild-type and TNF^{ARE/+} mice was similar for the first eight days following infection. There was no change in survival in either group until day 4, at which point there was a parallel decrease in survival for both groups of mice from days 5 through 8. However, beyond the 8th day the survival curves for the littermate control and TNF^{ARE/+} mice diverged. That is, whereas the survival curve for the littermate controls flattened out and showed no further increase in mortality, there was a continued progressive decrease in survival in the TNF^{ARE/+} mice from day 8 to day 14. Kaplan Meier analysis showed that there was a significant ($p < 0.05$) overall decrease in survival in the TNF^{ARE/+} mice (12.5%) when compared to littermate controls (45.8%) at day 14. However, when this analysis was confined to the first 7 days after EMCV infection, no significant difference was observed between the groups.

Myocardial inflammation in EMCV infected TNF^{ARE/+} mice

To determine the mechanism for the increased mortality in TNF^{ARE/+} mice, we assessed cardiac histopathology in littermate controls and TNF^{ARE/+} mice at 7 and 14 days after infection. As expected, there were no cellular infiltrates in the hearts of naive littermate control or TNF^{ARE/+} mice (Figure 2A). At 7 days there was a cellular infiltrate affecting ~25% of the myocardium in the littermate control and TNF^{ARE/+} mice (Figure 2B) [20]. However, the important finding shown by Figure 2 is that the degree of cellular infiltration at 14 days was significantly greater ($p < 0.05$) in TNF^{ARE/+} mice when compared with littermate control mice, consistent with the increased mortality observed in TNF^{ARE/+} mice at 14 days.

Viral replication in TNF^{ARE/+} mice

To determine whether the observed differences in survival and myocardial cellular infiltrates in the TNF^{ARE/+} mice were secondary to differences in viral replication, we measured EMCV viral genome by RT-PCR, and determined viral titers in hearts of EMCV infected mice. As shown in Figures 3A and 3B, EMCV genomic RNA levels were significantly lower ($p < 0.05$) in the hearts of TNF^{ARE/+} mice on day 7 when compared to littermate controls. Similarly, Figure 3C shows that the viral titers in the hearts of TNF^{ARE/+} mice ($1.17 \pm .30 \log_{10}$ PFU/mg) were significantly lower ($p < 0.05$) at day 7 when compared to littermate controls ($2.4 \pm .13 \log_{10}$ PFU/mg heart). Taken together, these data suggest that the TNF^{ARE/+} mice were able to eliminate replicating EMCV more efficiently than littermate control mice.

Myocardial proinflammatory cytokine and chemokine expression in TNF^{ARE/+} mice

Proinflammatory cytokines—To determine whether the increased myocardial inflammation in the TNF^{ARE/+} mice was related to increased cytokine expression, we examined TNF and IL-1 β gene and protein expression at baseline and days 3, 5, 7 and 14 after EMCV infection. Figure 4A shows that TNF and IL-1 β mRNA expression peaked 3 days after EMCV infection in littermate control mice, and then diminished towards baseline levels. Although the time course for the onset of TNF and IL-1 β mRNA expression in the hearts of TNF^{ARE/+} mice was similar to that observed in littermate controls, the duration of mRNA expression was significantly prolonged in TNF^{ARE/+} mice, consistent with the inability of the TNF^{ARE/+} mice to degrade TNF mRNA [15]. As shown by the representative RPA in Figure 4A and the group data summarized in Figures 4B-D, levels of TNF and IL-1 β mRNA were significantly increased in the TNF^{ARE/+} mice 7 days after EMCV infection when compared to littermate controls. Interestingly, there was no significant difference in the level of interferon- γ (ANOVA; $P = 0.98$) or interferon- β mRNA expression (ANOVA; $P = 0.2$.) between the two groups of mice (data not shown). Figures 4D and 4E show, respectively, that there was a 14-fold ($p < 0.05$) increase in TNF protein levels and a 6-fold ($p < 0.05$) increase in IL-1 β levels in the hearts of TNF^{ARE/+} mice relative to littermate controls.

Chemokines—Given that chemokines modulate recruitment of leukocytes to the site of viral infection,[25] we asked whether there were differences in the expression of chemokine mRNAs between EMCV-infected littermate control and TNF^{ARE/+} mice. Figure 5 shows that the kinetics of mRNA expression for RANTES, MCP-1, and IP-10 (Figure 5C) was similar in littermate control and TNF^{ARE/+} mice. However, the level of expression of these chemokines was significantly increased ($p < 0.05$) in the hearts of TNF^{ARE/+} mice, particularly on day 5 and 7 following EMCV infection. This finding is consistent with the histological analysis showing a higher level of leukocyte infiltration in the hearts of TNF^{ARE/+} mice. The mRNA expression of lymphotoxin- β , MIP-2, MIP-1 α and MIP-1 β did not differ between the two groups after EMCV infection (data not shown).

Role of innate and adaptive immune response in EMCV myocarditis

EMCV infected mice were treated with prednisolone from days 1 - 7 (early), in order to modulate the early innate immune response, or from days 7-14 (late) in order to modulate the late adaptive immune response.

Early Prednisolone Administration (days 1-7)—Figure 6 discloses two important findings with respect to the effect of early prednisolone administration on survival in EMCV infected littermate control and TNF^{ARE/+} mice. First, early administration of prednisolone to littermate controls resulted in increased mortality relative to untreated littermate controls ($p = 0.04$), consistent with a previous experimental report which showed that administration of prednisolone from days 4-13 was deleterious in a murine model of myocarditis [27]. Second, early administration of prednisolone to EMCV infected TNF^{ARE/+} mice resulted in improved survival relative to untreated TNF^{ARE/+} mice (36.4% vs. 12.5%, respectively; $p < 0.05$). Indeed, Figure 6A shows that the mortality curve for the prednisolone treated TNF^{ARE/+} mice was not significantly different ($p = 0.70$) from that observed in EMCV-infected untreated littermate control mice.

To explore the mechanisms for the above findings, we examined 7 day viral titers in the early prednisolone treated littermate control and TNF^{ARE/+} mice. Viral titers in the early prednisolone treated littermate controls ($3.2 \pm 0.2 \log_{10}$ PFU /mg tissue) were significantly greater ($p < 0.05$) than the viral titer in untreated littermate control mice ($2.4 \pm 0.13 \log_{10}$ PFU /mg tissue). Similarly, viral titers for the prednisolone treated TNF^{ARE/+} mice ($2.2 \pm 0.21 \log_{10}$ PFU /mg tissue) were significantly greater ($p < 0.05$) than the viral titers for the untreated TNF^{ARE/+} mice ($1.2 \pm 0.30 \log_{10}$ PFU /mg tissue), despite the fact that the mortality was greater in the untreated TNF^{ARE/+} mice. The dissociation between viral titer and survival was also evident in the comparison of treated TNF^{ARE/+} and the untreated littermate control mice, wherein the viral titers in the early prednisolone treated TNF^{ARE/+} ($2.2 \pm 0.21 \log_{10}$ PFU /mg tissue) mice were not significantly different from those in untreated littermate control mice ($2.4 \pm 0.32 \log_{10}$ PFU /mg tissue; $p = 0.6$; (Figure 3C), even though the mortality was greater in the early prednisolone treated TNF^{ARE/+}. To determine whether the dose of prednisolone used was sufficient to modulate the early innate immune response, we measured myocardial TNF protein levels at 7 days after infection in untreated and early prednisolone treated littermate control and TNF^{ARE/+} mice. Myocardial TNF protein levels in untreated littermate control mice were 3.6 ± 0.83 pg/mg protein while in the prednisolone treated littermate control mice TNF levels were below the detection of the assay. Myocardial TNF levels were also significantly reduced ($p < 0.0001$) in the early prednisolone treated TNF^{ARE/+} mice (16.8 ± 3.4 pg/mg protein) when compared to the untreated TNF^{ARE/+} mice (51.5 ± 16 pg/mg protein). However, the TNF levels were still greater than in the hearts of untreated and prednisolone-treated EMCV infected littermate control mice.

Late Prednisolone Administration (days 7-14)—The presence of inflammatory cell infiltration, including macrophages and T-cells, indicates that acquired immunity also plays an important role in the pathogenesis of myocarditis. To determine whether the observed increase in mortality at 14 days in EMCV infected TNF^{ARE/+} mice was the result of

increased activation of the adaptive immune response, as suggested by the increased cellular infiltration (see Figure 2), we treated EMCV infected littermate and TNF^{ARE/+} mice with prednisolone from day 7 to day 14 after infection. Figure 7A shows that although the rate of survival was improved in the prednisolone treated littermate controls relative to the untreated littermate controls, there was no overall significant difference between these two groups (58.8% vs. 45.8%, respectively; $p = 0.17$). In sharp contrast, Figure 7A shows that there was a significant ($p < 0.05$) improvement in 14 day survival in the late prednisolone treated TNF^{ARE/+} mice when compared to untreated TNF^{ARE/+} mice (41.2% vs. 12.5%). To address the potential mechanism(s) for the increased survival in the late prednisolone treated TNF^{ARE/+} mice, we examined cardiac histopathology in the untreated and late prednisolone treated TNF^{ARE/+} mice. There was significantly less ($p = 0.006$) cellular infiltration in the late prednisolone-treated TNF^{ARE/+} mice when compared to untreated TNF^{ARE/+} mice (Figure 7B and C). Indeed, the inflammatory score in the late prednisolone treated TNF^{ARE/+} mice at 14 days was not significantly different ($p = 0.5$) from the inflammatory score observed in the EMCV infected littermate control mice. Importantly, EMCV could not be recovered in culture from either untreated or late prednisolone treated TNF^{ARE/+} mice, further supporting the role of activation of the adaptive immune system in cardiac injury in the later stages of viral myocarditis. There were no differences in neutralizing antibody titers to EMCV in the late prednisolone treated and untreated EMCV-infected mice (data not shown).

DISCUSSION

Although previous studies have focused on the important role that acquired (adaptive) immunity plays in the pathogenesis of viral myocarditis [23], the role of the innate immune system in viral myocarditis is less well understood. The innate immune system plays an essential role as a primary sensor of invading pathogens, as well as in the induction of host antimicrobial defenses [1,14]. For example, following the recognition of viral proteins, effector cells initiate a rapid antiviral response that includes the brisk upregulation of a variety of inflammatory mediators such as TNF, NO, and interferon- β [11,14]. However, activation of the innate immune response in viral myocarditis may be a mixed blessing for the heart. That is, while activation of the innate response in the heart is beneficial to the host by virtue of its anti-viral effects, excessive or persistent activation of the innate immune system may lead to an exaggerated and/or chronic inflammatory process that triggers myocardial destruction and remodeling that culminates in cardiac dysfunction [6,33].

In the present study we examined the effect of EMCV infection in a unique line of mice (TNF^{ARE/+}) that are unable to downregulate TNF expression following infection. [15] The results of this study show for the first time that the duration and degree of activation of the innate immune system plays a critical role in determining host outcomes in EMCV viral myocarditis. Three major sets of experimental observations support this statement. First, sustained activation of the innate immune response in the TNF^{ARE/+} mice led to increased TNF synthesis (Figure 4) and decreased viral load in the heart (Figure 3), when compared to littermate controls. The critical role of the innate immune system was further shown by the studies in which early administration of prednisolone resulted in increased viral titer and increased mortality in wild-type mice (Figure 6). Second, the exaggerated innate immune

response in the TNF^{ARE/+} mice resulted in increased myocardial inflammation (Figure 2) and increased late mortality when compared to wild type mice (Figure 1). The increased inflammation in the TNF^{ARE/+} mice was accompanied by increased and prolonged expression of the chemokines RANTES/CCL5, MCP-1 and IP-10/CXCL10 in the myocardium (Figure 5), which are known to activate and interface with the acquired immune system by modulating the migration of immune cell populations such as macrophages and T-lymphocytes into the myocardium [9]. Third, late administration of prednisolone resulted in a significant improvement in survival and decreased cardiac inflammation in the TNF^{ARE/+} mice by attenuating the acquired immune response following EMCV infection (Figure 7). Taken together, these data suggest that activation of the innate immune system plays an important role in the early host response to viral infection by limiting viral load, and that sustained activation of the innate immune system can contribute to myocardial damage through excessive activation of the acquired immune system, which results in increased myocardial inflammation

As noted above, the adaptive immune system (particularly CD4⁺ and CD8⁺ T-cells) plays a critical role in the pathogenesis of viral myocarditis. Opavsky et al.[23] reported increased survival and decreased myocardial damage after viral infection in CD4^{-/-}CD8^{-/-} mice. Interestingly, myocardial viral clearance was not impaired in the CD4^{-/-}CD8^{-/-} mice when compared to wild type mice, suggesting that both CD4⁺ and CD8⁺ T cells participate in cardiac tissue damage but do not influence viral titer. Based on these prior observations, we reasoned that the increased mortality and myocardial inflammation in the TNF^{ARE/+} mice might be secondary, at least in part, to the excessive activation of the adaptive immune system. To address this question we treated TNF^{ARE/+} mice with the immune modulating agent prednisolone. The early administration of prednisolone to EMCV infected TNF^{ARE/+} mice resulted in improved survival relative to untreated TNF^{ARE/+} mice, as well as decreased synthesis of myocardial TNF production. Importantly, viral clearance was not impaired in the prednisolone treated TNF^{ARE/+} mice. Moreover, administration of prednisolone to TNF^{ARE/+} mice on days 7 to 14 after EMCV infection significantly improved the 14-day survival rate compared to untreated TNF^{ARE/+} mice. The improved survival in the prednisolone treated TNF^{ARE/+} mice was associated with a significant decrease in myocardial inflammation, consistent with the prevailing notion that innate immune mechanisms are important in modulating key aspects of the adaptive immune response. As a case in point, Fairweather et al.[7] recently reported that mice with defective Toll-like receptor 4 (TLR4) signaling had significantly reduced levels of myocarditis (inflammation) and viral replication 12 days after infection when compared to wild type mice. Cardiac levels of IL-1 β and IL-18 were reduced in TLR4 deficient mice at 12 days suggesting that innate immune mechanism contributed to the pathogenesis of myocarditis. Interestingly, 2 days after infection viral titers were significantly higher in the hearts of TLR4 deficient mice when compared to wild type littermates. Although this study concluded that activation of the innate immune system had a significant deleterious effect on the heart in the late stages of viral infection, it also suggested an important protective role for this system in the acute stages of viral heart disease.

The present study provides an important clarification with respect to the role of proinflammatory cytokines in viral myocarditis. In the present study, TNF levels were

significantly higher (14-fold) in the hearts TNF^{ARE/+} than in wild type mice following EMCV infection. Interestingly, TNF^{ARE/+} mice had 18-fold less EMCV in their hearts than wild type mice under comparable conditions of infection, suggesting that endogenously produced TNF (or molecules that are downstream from TNF) inhibits EMCV replication *in vivo*. Indeed, previous studies have shown that gene targeted deletion of TNF or NO adversely affects the course of viral myocarditis [28,32]. Relevant to the present discussion, there was an increase in myocardial viral load in TNF or NO knockout mice. Further, studies in TNF^{-/-} mice have shown that treatment with recombinant human TNF improves survival after EMCV infection [28]. Although viral titers were not determined after treatment in this study, our results suggest that the improvement in survival may have been secondary to the inhibitory effects of TNF on EMCV replication. Additional evidence confirming the beneficial effect of early TNF production in viral myocarditis has been provided by Tanaka et al [26]. In this study, cardiac overexpression of IL-6 led to accelerated myocardial injury and decreased viral clearance after EMCV infection, which was thought to be secondary a decrease in circulating levels of TNF. Thus, the extant literature suggests that the delicate balance between the beneficial antiviral effect of cytokines and the deleterious effects associated with cytokine-induced inflammatory responses, contributes importantly to host outcomes following viral infection.

Conclusion

The results of this study suggest that activation of the innate immune system plays an important role in the early host response to viral infection by controlling viral replication, whereas sustained activation of the innate immune response can contribute to myocardial damage through excessive activation of the acquired immune system. Although it is not possible to directly extend these experimental studies in mice to clinical observations in humans, the results of the present study do offer one potential explanation for why prior clinical studies in patients with myocarditis may have failed to show benefit [19]. That is, many studies in patients with myocarditis have employed steroids or other immune modulating agents to suppress myocardial inflammation. While this strategy effectively suppresses the deleterious actions of excessive activation of the adaptive immune system, it does so at the expense of suppressing the beneficial effects of the innate immune system on viral load, which has been implicated in the pathogenesis of dilated cardiomyopathy [3-5]. Germane to this discussion, a recent provisional report suggests that immunosuppressive therapy is beneficial in patients with myocarditis, who have negative viral titers. Thus, the results of the present study may have direct clinical relevance [30].

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Figure 1.

TNF overexpression decreases survival after EMCV infection. Littermate controls and TNF^{ARE/+} mice were inoculated intraperitoneally with 500 pfu EMCV. TNF^{ARE/+} mice had significantly reduced survival at 14 days. *P*-values obtained using a Log rank test. * *P* < 0.05. Littermate controls vs. TNF^{ARE/+} mice.

Figure 2. Histological analysis of EMCV-infected heart tissues. (A) Representative hematoxylin and eosin-stained sections of hearts from littermate and TNF^{ARE/+} mice harvested on day 14 after EMCV infection (magnification x20). (B) Myocardial histopathological scores at day 14 after infection. *P < 0.05

Figure 3.

TNF overexpression decreases EMCV replication. (A) Representative semi-quantitative RT-PCR analysis of viral genomic RNA in cardiac tissue at day 7 after infection. (B) EMCV RNA levels normalized to β -actin mRNA. (C) Viral titers in hearts harvested from littermate and $TNF^{ARE/+}$ mice at 14 days after infection. Results are expressed as mean $\pm$ SEM. * $P < 0.05$

Figure 4.

Effect of EMCV infection on cardiac TNF and IL-1 β mRNA expression (A) TNF and IL-1 β IL-6 mRNA expression was assessed by RPA. TNF (B) and IL-1 β (C) mRNA levels normalized to L32. *, $p < 0.05$ Littermate vs. TNF^{ARE/+} mice. Cardiac TNF (D) and IL-1 β (E) protein levels. *, $p < 0.05$; littermate vs. TNF^{ARE/+} mice.

Figure 5.

Cardiac RANTES, MCP-1 and IP-10 mRNA expression after EMCV infection. (A) Cardiac RANTES, MCP-1 and IP-10 mRNA expression was assessed by RPA. RANTES (B), MCP-1(C) and IP-10 (D) mRNA levels normalized to L32. *, $P < 0.05$; Littermate control vs. TNF^{ARE}^{+/+} mice.

Figure 6.

Early prednisolone treatment improves 14-day survival of TNF^{ARE/+} mice infected with EMCV. Littermate controls and TNF^{ARE/+} mice were inoculated intraperitoneally with 500 pfu EMCV. TNF^{ARE/+} mice and littermates were treated with prednisolone as described in text. *P*-values obtained using a Log rank test. * *P* < 0.05; littermate controls vs. TNF^{ARE/+} mice.

Figure 7.

Late prednisolone treatment improves 14-day survival and decreases severity of myocarditis in TNF^{ARE/+} mice infected with EMCV (A). Littermate controls and TNF^{ARE/+} mice were inoculated intraperitoneally with 500 pfu EMCV. TNF^{ARE/+} mice and littermates were treated with prednisolone at described in text. *P*-values obtained using a Log rank test.

* *P* < 0.05; littermate controls vs. TNF^{ARE/+} mice. (B) Representative hematoxylin and eosin-stained sections of hearts from TNF^{ARE/+} mice harvested on day 14 after EMCV infection and late prednisolone treatment (magnification x20). (C) Myocardial histopathological scores at day 14 after infection and late prednisolone treatment. **P* < 0.05