Myeloid Differentiation Factor-88 Plays a Crucial Role in the Pathogenesis of Coxsackievirus B3–Induced Myocarditis and Influences Type I Interferon Production

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Background—Myeloid differentiation factor (MyD)-88 is a key adaptor protein that plays a major role in the innate immune pathway. How MyD88 may regulate host response in inflammatory heart disease is unknown.

Methods and Results—We found that the cardiac protein level of MyD88 was significantly increased in the hearts of wild-type mice after exposure to Coxsackievirus B3 (CVB3). MyD88−/− mice showed a dramatic higher survival rate (86%) in contrast to the low survival (35%) in the MyD88+/+ mice after CVB3 infection (P<0.0001). Pathological examination showed a significant decrease of cardiac and pancreatic inflammation in the MyD88−/− mice. Viral concentrations in the hearts were significantly decreased in the MyD88−/− mice. Cardiac mRNA levels for interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-18 were significantly decreased in the MyD88−/− mice. Similarly, serum levels of T-helper 1 cytokines were significantly decreased in the MyD88−/− mice. In contrast, cardiac protein levels of the activated interferon regulatory factor (IRF)-3 and IFN-β were significantly increased in the MyD88−/− mice but not other usual upstream signals to IRF-3. The cardiac expression of coxsackie-adenoviral receptor and p56lck were also significantly decreased.

Conclusions—MyD88 appears to be a key contributor to cardiac inflammation, mediating cytokine production and T-helper-1/2 cytokine balance, increasing coxsackie-adenoviral receptor and p56lck expression and viral titers after CVB3 exposure. Absence of MyD88 confers host protection possibly through novel direct activation of IRF-3 and IFN-β. (Circulation. 2005;112:2276-2285.)

Key Words: heart failure ■ immunology ■ inflammation ■ myocarditis ■ viruses

Viral myocarditis is one of the important causes of acute and chronic heart failure.1 The spectrum of disease spans from a fulminant course to mild symptoms and has been linked to the development of dilated cardiomyopathy.2 Mice infected with Coxsackievirus B3 (CVB3) result in a disease similar to the clinical heart disease observed in human beings, with the development of acute myocarditis from day 7 to 14 after infection that later progress to a chronic, autoimmune phase of disease.3 This model is valuable in studying the disease pathogenesis. We have dissected out the contribution of the acquired immunity through T-cell receptor signaling toward the disease.4 We have identified the role of host T-cell–associated immune tyrosine kinase p56lck and its phosphatase CD45,5 as key molecules responsible for the trigger and consequences of the inflammatory response in the disease. In our earlier investigation, the acquired immunity responses occur relatively late after the initial viral infection, and much of the initial host response to the viral infection involved key signals that trigger the innate immunity. However, innate immunity has two faces in that on the one hand it turns on host defending antiviral agents such as interferons (IFNs) and on the other hand can also trigger late acquired immunity. How innate immunity is triggered and how it plays a role of pathogenesis in CVB3 infection is not completely understood.

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Innate immunity probably plays an important role in general cardiovascular condition such as atherosclerosis, postinfarct healing and remodeling, and heart failure. One of the mechanisms by which the innate immune system senses the presence of foreign antigens is through the Toll-like receptors (TLRs), which recognize generalized molecular patterns.7,8 TLRs are type 1 transmembrane receptors that...
have extracellular leucine-rich repeat domain and cytoplasmic domain homologous to interleukin-1 receptor (IL-1R). On molecular pattern recognition, most of all TLRs recruit the IL-1R–associated kinases (IRAKs) through the adapter molecule myeloid differentiation factor-88 (MyD88), which in turn activates nuclear factor-κB (NF-κB) through tumor necrosis factor receptor–associated factor 6 (TRAF6) and other intermediates. MyD88 originally was isolated as a myeloid differentiation primary response gene and has been shown to act as an adaptor molecule that plays an important role in TLR/IL-1R signaling. Immunization and infection studies using MyD88-deficient (MyD88−/−) mice have revealed that MyD88 is critical for the activation of innate immunity and host defense. Macrophages and dendritic cells from MyD88−/− mice fail to produce inflammatory cytokines in response to a variety of pathogen-associated molecular patterns. MyD88−/− mice are resistant to lipopolysaccharide-induced endotoxin shock. On the other hand, MyD88−/− mice have been shown to be highly susceptible to a number of bacterial pathogens, including Listeria monocytogenes, Staphylococcus aureus, and Toxoplasma gondii, and Mycobacterium avium. However, the role of MyD88 in the pathogenesis of viral infection in general and myocarditis in particular, and the association between MyD88 and IFN-induced antiviral activity, are still not clear. This constitutes the objectives of this study.

Methods

Virus

The cardiovirulent strain of CVB3 (Charles Gauntt; CG) was prepared by passage through HeLa cell cultures and then titrated by plaque assay and stored at −80°C. Aliquots from the same stock were used for all animals.

Mice

MyD88−/− mice with the genetic background of C57BL/6J were generated and maintained as described previously. After heterozygous (+/−) mating, heterozygous (+/−), homozygous (−/−), and wild-type (+/+), mice were identified by PCR analysis of DNA obtained from the tail of each mouse. Wild-type C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The study was performed in accordance with the policies of the Animal Care Committee of the Toronto General Hospital. C57BL/6 wild-type (n=60) and MyD88−/− mice (n=50) ages 6 to 8 weeks were inoculated intraperitoneally with 10⁵ plaque-forming units (PFU) of CVB3 on day 0. Animals were observed for spontaneous death until day 14, and a subgroup was randomly assigned to euthanasia on 4, 7, 10, and 14 days after infection. The animals that were randomized for euthanasia were censored from the mortality data.

Figure 1. Cardiac expression of MyD88 and IRAK-4 by Western blot analysis in C57BL/6 wild-type mice infected with CVB3 (A). There was faint baseline presence of the protein; however, the levels of MyD88 (B) and IRAK-4 (C) increased quickly and persisted even beyond day 10. Values are expressed as mean±SEM (n=5 per group) *P<0.05 vs day 0.
Histopathology

Transverse midsections of hearts were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and processed for hematoxylin and eosin staining. Histopathologic grading of cellular infiltrate and necrosis of the myocardium was evaluated by 2 independent observers in a blinded manner. The sections were rated on a scale of 0 to 4, as previously published. Paraffin-embedded pancreases were also examined for evidence of inflammation and necrosis after CVB3 infection.

Viral Titers

After aseptic removal, hearts, livers, and spleens were stored individually at −80°C. Organ samples were weighed and homogenized in 2 mL of RPMI-1640. After 3 freeze-thaw cycles and
Measurement of Serum Cytokines

Serum levels of IFN-γ, tumor necrosis factor (TNF)-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12 were determined by multiplex bead-based cytokine assay, using the LiquiChip System (QIAGEN). Whole cardiac cell lysates were mixed with 2× Tris-glycine SDS sample buffer (Invitrogen), boiled, and proteins resolved by SDS-PAGE, using 8% to 16% gradient Tris-Glycine gel (Invitrogen), and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour at room temperature with 5% powdered skim milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST), reacted with anti–IRAK-4 antibody (Upstate Biotechnology Inc), anti–interferon regulatory factor (IRF)-3 antibody (Santa Cruz), anti–TANK-binding kinase (TBK)-1 antibody (Santa Cruz), anti-Coxsackie-adenoviral receptor (CAR) antibody (Santa Cruz), anti-p56lck antibody (Santa Cruz), and anti–I-kappa-B kinase (IKK)-i (Santa Cruz). After membranes were incubated in stripping buffer (10 mmol/L 2-mercaptoethanol, 2% wt/vol SDS, 62.5 mmol/L Tris, pH 6.7) for 30 minutes at 55°C, they were washed in TBST, blocked, and reacted with anti–IRF-3 antibody. The quantification of the dimer of IRF-3 was performed with NIH Image software after expression as a ratio to the monomer of IRF-3 for the same sample.

Statistical Analysis

Results are expressed as mean±SEM. Survival was analyzed by the Kaplan-Meier method, and survival differences between groups were tested by log-rank test. Comparisons between the groups were performed by one-way ANOVA, followed by the Bonferroni-Dunn post hoc testing method. A value of P<0.05 was considered statistically significant.

Figure 3. Viral titers of hearts (A), livers (B) and spleens (C) in the MyD88+/− (WT) mice (●) and MyD88−/− (KO) mice (○) infected with CVB3. Viral titers were determined from total organ homogenates, using plaque assays on HeLa cell monolayers; n=5 mice per group analyzed for each time point. Data represent mean values of CVB3 PFU per gram of tissue, to normalize for organ size. Viral concentrations of hearts, livers, and spleens were significantly decreased in MyD88 KO mice compared with WT. D, Neutralizing antibody titers after CVB3 infection. Sera were collected from day 7 to day 10 after infection, inactivated, and serially diluted 1:2 to determine the highest dilution able to completely inhibit CPE by CVB3. Titers are expressed as reciprocal dilutions of serum. No significant differences in neutralizing antibody titers were found between WT mice and MyD88 KO mice. ▴P<0.05 vs WT mice.
Results
Expression of MyD88 and IRAK-4 in Mice With CVB3-Induced Myocarditis
The innate immune response triggered by CVB3 infection remains uncharacterized. We hypothesized that TLR signals play a role in initiation of antiviral response. To investigate this hypothesis, we first examined the protein levels of two important key signaling molecules downstream of TLRs, namely MyD88 and IRAK-4 in the hearts of mice after inoculated CVB3. We quantified the protein levels of MyD88 and IRAK-4 by using Western blot analysis (Figure 1A). We could detect the expression of MyD88 and IRAK-4 in normal
hearts of mice. The cardiac protein levels of MyD88 (Figure 1B) and IRAK-4 (Figure 1C) were significantly upregulated after inoculation with CVB3 in wild-type mice. These results suggest that these signaling proteins are present at all times but become further enriched on CVB3 infection.

**MyD88**−/− Mice Are Protected From Acute CVB3 Infection

MyD88 is necessary for production of inflammatory cytokines but is not necessary for antiviral responses in TLR4 signaling. However, MyD88 appears to be an important adapter for signaling, including NF-κB and IRF-7 in TLR7, TLR8, and TLR9. On the other hand, if other TLRs, such as TLR3, which activate IRF-3 and type I IFNs, are also used to fight against virus, the deficiency of MyD88 and the dominance of MyD88-independent pathway in CVB3 infection would show a better outcome. We infected MyD88−/− mice with CVB3, which showed a dramatic higher 14-day survival rate at 86% in contrast to the low survival rate of 35% in the MyD88+/+ mice (P<0.0001) (Figure 2A). The maximum mortality rate took place between 3 to 7 days after inoculation, corresponding to the acute phase of the infection. Kaplan-Meier survival analysis showed a significant difference between the two groups. In the MyD88+/+ mice infected with CVB3, heart weight/body weight ratio increased significantly compared with MyD88−/− mice on days 10 and 14 (Figure 2B), suggesting significant cardiovascular remodeling. The levels of blood sugar, which is one of the peripheral markers of stress, increased significantly in the MyD88+/+ compared with MyD88−/− animals on days 4, 5, and 6 (Figure 2C). After CVB3 infection, cardiac pathology was consistent with observed mortality rates. Widespread myocardial infiltration of inflammatory cells and myocyte necrosis were observed in the MyD88+/+ mice on days 7, 10, and 14. On the other hand, infiltration was mild to absent in MyD88−/− mice (Figure 2, D, E, and H). In the pancreas, moderate to severe inflammation was observed in the MyD88+/+ mice. However, there was only minimal to mild inflammation in MyD88−/− mice (Figure 2, F and G).

**Viral Titters Correlated With Severity of Myocarditis and Mortality Rates**

To examine whether the differences in survival rate and pathology were due to the viral replication, CVB3 viral titers from hearts, livers, and spleens were assessed by plaque assay. The amount of infectious CVB3 was significantly decreased in the hearts, livers, and spleens of MyD88−/− mice as compared with their wild-type littermates (Figure 3, A, B, and C). Infectious CVB3 was no longer detectable by 10 days after viral inoculation in MyD88−/− mice. Thus, MyD88 is important for CVB3 replication, CVB3 persistence, and the pathogenesis of CVB3-mediated disease in vivo. The neutralizing antibody response, which is representative of B-cell activation, did not show any significant difference between MyD88+/+ and MyD88−/− mice (Figure 3D).

**MyD88-Regulated Cytokine Balance**

TLRs lead to IRF 3/7-type I IFNs but can also activate inflammatory cytokines such as IL-1 and TNF-α and antiinflammatory cytokines such as IL-10. In the pathogenesis of viral myocarditis, this cytokine balance is important. To determine how MyD88 affects the cytokine response in myocarditis, cardiac cytokine RNA expression was evaluated by semiquantitative RT-PCR. Cardiac RNA levels of IL-1β, TNF-α, IFN-γ, IL-10, and IL-18 were significantly decreased in MyD88−/− mice. No significant difference of inducible form of nitric oxide synthase between genotypes was observed. By contrast, cardiac RNA levels of IFN-α and IFN-β were significantly increased in MyD88−/− mice (Figure 4). Serum levels of IL-1β, TNF-α, IFN-γ, IL-2, IL-6, and IL-12 at day 7 were significantly decreased in MyD88−/− mice. Interestingly, IL-4 and IL-10, which are representative T-helper 2 cytokine profile, showed no significant differences between both groups (Figure 5).

**MyD88-Influenced Expression of Other Components of Innate and Acquired Immunity Pathway**

How MyD88 deficiency plays a role of pathogenesis and influences other innate immune components, especially the MyD88-independent pathway in CVB3 infection, is not understood. Furthermore, data to date suggest that innate immunity appears to be required for the proper induction of acquired immunity. In this study, the cardiac protein level of IRAK-4 was significantly decreased in the MyD88−/− mice (Figure 6A); however, those of IRF-3 (Figure 6B) and activated IRF-3 (dimeric form of IRF-3) (Figure 6C) were increased significantly compared with the MyD88−/− mice. Furthermore, the cardiac protein level of IFN-β (Figure 6D) was also significantly increased in the MyD88−/− mice. In contrast, the expression of IKK-i and TBK-1, which are upstream of IRF-3, was decreased in the MyD88−/− mice (Figure 6, E and F). On the other hand, the cardiac protein levels of CAR (Figure 6G) and T-cell costimulatory tyrosine kinase, p56lck (Figure 6H), which is required for efficient CVB3 replication, were significantly decreased in MyD88−/− mice after exposed to CVB3.

**Discussion**

In this study, we examined the role of MyD88 on the development of acute CVB3-induced myocarditis, using MyD88-deficient mice. We report that MyD88 deficiency results in decreased viral replication and inflammation in the heart. Consistent with this observation was the decrease in the proinflammatory cytokines in the heart, with a first-time observation of a concomitant upregulation of IRF-3 and IFN-β levels after CVB3 infection in the MyD88 null mice.

It is well established that TLRs play a crucial role in the recognition of microbial pathogens and inducing innate immune responses in mammalian hosts. Although the majority of the work on TLRs has focused on detection of bacteria, it is becoming increasingly apparent that viruses are also subject to innate sensing and processing by TLRs. However, the signaling and response may differ between viruses and bacteria. MyD88−/− mice have been shown to be highly susceptible to a number of nonviral pathogens. On the other hand, Fairweather et al have recently evaluated CVB3 infection in TLR4-deficient mice. They identified that viral
TLR4 is capable of recognizing some viruses.26,27 Recently, the mice and MyD88 inducing IFN-α, β, and IL-18. These results suggest that MyD88-dependent pathways may play a different role between bacterial and viral infection.

MyD88 plays essential role for all TLR-mediated production of inflammatory cytokines. Two exceptions are TLR3 and TLR4. Previous studies with mice that have a targeted disruption of the MyD88 gene have revealed that TLR3 and TLR4 use both MyD88-dependent and MyD88-independent pathways to initiate the innate immunity response.25 It was also reported that TLR4 is capable of recognizing some viruses.26,27 Recently, the ligands for TLR7 and TLR8 were identified as single-stranded RNA. Like TLR9 (CpG DNA triggered), TLR7 and TLR8 require endosome acidification for proper activity and induce type I IFN production in a MyD88-dependent manner. Thus, these three TLRs share similar mechanisms of action to recognize and respond to distinct nucleic acid targets.28–31 Furthermore, MyD88 is the sole adapter for TLR7, TLR8, and TLR9 and that is responsible for both NF-κB or inflammatory cytokines and IRF activation.32,33

Current understanding of the innate immunity signaling system in viral infection suggests that the ultimate outcome of innate immunity activation rests on the relative degrees of activation of the MyD88-dependent pathway, which is the MyD88–IRAKs–TRAF6–NF-κB–inflammatory cytokine arm versus the MyD88-independent pathway, which is the TRIF–IRF-3/7–type I IFN arm. As the TLRs that are most relevant for viral infection include TLR3, TLR4, TLR7, TLR8, and TLR9, the most relevant receptor adaptors are indeed MyD88 and TRIF. We therefore hypothesize that the balance of activation between MyD88-dependent pathway and MyD88-independent pathway may represent the key decision for making or modulation point of the protective and harmful aspects of the innate immunity in CVB3 infection.

Viral myocarditis is now recognized as a triphasic disease involving an initial viral proliferative component, followed by host immune response including autoimmune perpetuation, and finally remodeling of the cardiac structure and function.4 Optimal outcome is achieved by effective attenuation of viral proliferation and appropriate host immune response. Our results show that MyD88−/−, animals when exposed to CVB3, have predominant IRF-3 and IFN-β activation, accompanied by less inflammatory cell infiltration, decreased viral proliferation, and death. This suggests that the MyD88-dependent pathway contributes pathophysiologically in the CVB3 replication during early phase of the disease and the production of proinflammatory cytokines to maintain the late phase of the disease. In addition, this is the first report that the deficiency of MyD88 influences the MyD88-independent pathway. In this study, MyD88 deficiency did not influence the expression of IKK-i and TBK-1, which are upstream of IRF-3 and downstream of TRIF after CVB3 infection. MyD88 may thus be able to directly regulate IRF-3 during a viral infection. These results suggest that there is a hitherto unrecognized novel cross-talk between MyD88 and IRF-3, formally through to be part of a MyD88-independent signaling pathway (Figure 7).

On the other hand, our results also show for the first time that MyD88 pathway influences the expression of the critical CAR in the process of CVB3 infection. We have previously identified that the CVB3 virus enters the target cells through two distinct but collaborating receptors—CAR, responsible for internalization, and decay accelerating factor, responsible for virus-CAR interaction. The main internalizing receptor is CAR, which we have demonstrated to be important for all coxsackie viruses to gain tissue entry, and is a member of the immunoglobulin superfamily with adhesion properties.34,35 The expression of CAR has been previously reported to be increased in association with myocarditis and cardiomyopathy.36,37 We also have identified a variety of splice variants of CAR molecules that correlates with host susceptibility.34,38

Figure 5. Serum levels of various cytokines in MyD88−/− (WT) mice and MyD88−/− (KO) mice (5 mice/each time point) were analyzed by multiplex bead-based cytokine assay. *P<0.05 vs WT mice.
We have identified previously that p56\textsuperscript{ck}, the T-cell receptor associated tyrosine kinase, is critical for both virus proliferation in the heart and activation of the T cells to maintain an inflammatory response in the heart.\textsuperscript{5} We have also reported that the absence of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell subsets conferred a beneficial effect after CVB3 infection.\textsuperscript{4} The same benefit was observed in TCR-\textsuperscript{β--}deficient mice infected with CVB3. In our study, we observed that MyD88 deficiency also led to significant reduction in p56\textsuperscript{ck} expression and inflammatory cell infiltrate. Taken together, this suggests an important linkage between the MyD88-dependent innate immune response and subsequent activation of the acquired immune system, including the critical p56\textsuperscript{ck} pathway for T-cell activation. This supports the evolving concept that the acquired immune system does not function independently and that almost every aspect of the acquired immune system may be controlled by the innate immune system.\textsuperscript{30}

To summarize, we propose that the MyD88 adapter signal is a major contributor to cardiac inflammation after CVB3 exposure (Figure 7). MyD88 can activate downstream inflammatory signals including IRAK-4, TRAF6, and nuclear translocation of NF-\kappaB, leading to increased cardiac cytokine production such as IL-1\beta, TNF-\alpha, and IFN-\gamma. In addition, MyD88 increases tissue CAR expression, leading to increased viral entry, viral proliferation, and increased viral injury. This combination leads to increased viral titers in the heart, increased inflammation, and resulting cardiac damage from both loss of myocytes and cytokine-induced dysfunction. However, during MyD88 deficiency, not only are the downstream cytokines reduced and the viral titers and viral

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\caption{Cardiac protein expression of IRAK-4 (A) was significantly decreased; however that of IRF-3 (B) and activated IRF-3 (analyzed by native PAGE) (C) were significantly increased in MyD88\textsuperscript{−/−} (KO) mice compared with MyD88\textsuperscript{+/+} (WT) mice at day 7 after CVB3 inoculation. The cardiac protein level of IFN-\beta (D) in KO mice was significantly increased at day 4 after CVB3 inoculation. Expression of IKK-i was not different between both groups (E), and TBK-1 was decreased in KO mice (F). The cardiac protein expression of CAR (G) and p56\textsuperscript{ck} (H) in KO mice was significantly decreased at day 7 after CVB3 inoculation. Data represent mean\textpm SEM for 5 mice per group. *\textit{P}<0.05 vs WT mice.}
\end{figure}
manipulation of the MyD88-regulated pathway to rebalance the host inflammatory response to viral exposure. Manipulation of the MyD88-regulated pathway to rebalance the host immune response may represent a novel therapeutic opportunity.

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References


CLINICAL PERSPECTIVE

Innate immunity mediates early host response to external pathogens or dangerous stresses without the need of antigenic specificity. A major gateway to innate immunity activation is through the family of Toll-like receptors that are ubiquitously present on all cell surfaces, including the cardiovascular system. Innate immunity members such as Toll-like receptors are critical for host defense, but on the other hand can trigger inflammation through the amplification of antigen-specific T and B cells as part of the acquired immunity response. We demonstrated in a coxsackie viral myocarditis model that the common toll-receptor adaptor MyD-88 may play a critical proinflammatory role in the host response to viral injury. Genetic removal of MyD-88 led to improved survival, pathology, lower viral titers, inflammatory cytokines, and less T-cell activation. However, there was in contrast higher levels of protective type I interferon and its transcriptional control: interferon regulatory factor-3. This study suggests that there are critical signal switches within the innate immune response in the cardiovascular system. Appropriate understanding of this system may help to identify the key points for therapeutic manipulation in the future to modulate the inflammatory response to external pathogen or stresses.
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