Myeloid Differentiation Factor-88/Interleukin-1 Signaling Controls Cardiac Fibrosis and Heart Failure Progression in Inflammatory Dilated Cardiomyopathy

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Rationale: The myeloid differentiation factor (MyD88/interleukin (IL)-1 axis activates self-antigen-presenting cells and promotes autoreactive CD4+ T-cell expansion in experimental autoimmune myocarditis, a mouse model of inflammatory heart disease.

Objective: The aim of this study was to determine the role of MyD88 and IL-1 in the progression of acute myocarditis to an end-stage heart failure.

Methods and Results: Using α-myosin heavy chain peptide (MyHC-α)-loaded, activated dendritic cells, we induced myocarditis in wild-type and MyD88−/− mice with similar distributions of heart-infiltrating cell subsets and comparable CD4+ T-cell responses. Injection of complete Freund’s adjuvant (CFA) or MyHC-α/CFA into diseased mice promoted cardiac fibrosis, induced ventricular dilation, and impaired heart function in wild-type but not in MyD88−/− mice. Experiments with chimeric mice confirmed the bone marrow origin of the fibroblasts replacing inflammatory infiltrates and showed that MyD88 and IL-1 receptor type I signaling on bone marrow–derived cells was critical for development of cardiac fibrosis during progression to heart failure.

Conclusions: Our findings indicate a critical role of MyD88/IL-1 signaling in the bone marrow compartment in postinflammatory cardiac fibrosis and heart failure and point to novel therapeutic strategies against inflammatory cardiomyopathy. (Circ Res. 2009;105:912-920.)

Key Words: autoimmune myocarditis ■ heart failure ■ fibrosis ■ innate immunity

Inflammatory cardiomyopathy refers to a subtype of dilated cardiomyopathy, a common cause of heart failure in young adults.1 Inflammatory cardiomyopathy often results from infection-triggered myocarditis and is characterized by impaired cardiac contractility, ventricular dilation, and progressive myocardial fibrosis. Many viruses but also bacteria, parasites, drugs, and systemic diseases are supposed to trigger cardiac inflammation. It is not clear yet how these different etiologic agents promote the progression of acute cardiac inflammation to a common final pathway of end-stage heart failure with a common morphological phenotype.

Clinical observations and animal experiments suggest that autoimmunity plays an important role in inflammatory cardiomyopathy. In the experimental autoimmune myocarditis (EAM) model, disease development depends on the sequential activation of self–antigen-presenting cells (APCs) and the expansion of autoreactive, heart-specific CD4+ T cells.7–11 We found that cardiac damage of any cause, together with nonspecific activation of APCs via Toll-like receptors (TLRs) is sufficient to break immunotolerance.12,13 Accordingly, activated bone marrow–derived dendritic cells (bmDCs) loaded with α-myosin heavy chain (MyHC-α) peptide induce acute CD4+ T cell–mediated myocarditis in susceptible mice.12,13 IL-1 receptor and most of the TLRs share the common downstream signaling adaptor molecule MyD88.14,15 MyD88−/− and IL-1 receptor (IL-1R) signaling on APCs are critical for the induction of heart-specific T-cell responses and EAM. However, adoptive transfer of wild-type bmDCs with intact IL-1R/MyD88 signaling restores myocarditis susceptibility of MyD88−/− and IL-1R−/− mice.13,16

These observations might explain why different etiologic agents can induce acute cardiac inflammation in susceptible individuals. However, it does not explain how pathological remodelling and dilated cardiomyopathy develop after resolution of inflammation in some of the affected hearts. In humans, dilation of the left ventricle, and cardiac fibrosis are
hallmarks of end-stage congestive heart failure. Enhanced cardiac fibrosis results in a restrictive diastolic filling pattern, which is indicative of a poor prognosis. In the EAM model, similar to the observations in humans, myocarditis largely resolves and some mice develop fibrosis, ventricular dilation, and impaired fractional shortening.12,17–19

Here, we provide evidence that progression of EAM into an end-stage heart failure phenotype depends on the sequential activation of MyD88- and IL-1R–dependent signaling pathways in the inflammatory bone marrow (BM)-derived cell compartment. Furthermore, we introduce a novel approach to induce accelerated heart failure and cardiac fibrosis in a mouse model of inflammatory heart disease.

Methods

Mice

BALB/c (n = 125), MyD88−/− (n = 60), IL-1R−/− (n = 17) mice (maintained on a BALB/c background as described previously13,16) and C57BL/6-EGFP transgenic mice (expressing enhanced green fluorescent protein [EGFP]) under the control of the B-actin promoter) backcrossed into BALB/c background for >10 generations (hereafter referred to as BALB/c-EGFP, n = 8) were used at 6 to 8 weeks of age. Animal experiments were performed in accordance with the Swiss federal law and were approved by the local authorities.

Chimeric Mice

Six- to 8-week old mice were lethally irradiated with 2 doses of 6.5 Gy as described11 and transplanted with total of 2×107 crude BM cells from BALB/c, BALB/c-EGFP, MyD88−/− or IL-1R−/−. Chimeric mice were used 6 weeks after BM reconstitution.

Immunization Protocols

Myocarditis was induced by bmDCs, as described.12,13 Briefly, immature bmDCs were pulsed with 10 μg/mL MyHC-α614–634 (Ac-SLKLMATLFSTYASAD-OH) or ovalbumin (OVA 323–339) peptide, activated with 0.1 μg/mL lipopolysaccharide and 5 μg/mL anti-CD40 before intraperitoneal injection of 5×106 cells per mouse at days 0, 3, and 5. In the respective experiments, mice received additional subcutaneous injections of 200 μL of PBS or complete Freund’s adjuvant (CFA) emulsified with PBS (1:1) with or without 100 μg of MyHC-α at days 10 and 17. In some experiments, bmDC-immunized mice were injected with 1 μg/mL lipopolysaccharide (Escherichia coli 0111:B4; Sigma), 10 μmol/L CpG-ODN, or 10 μg/mL peptidoglycan (Staphylococcus aureus; Fluka) every second day, repetitively, for 14 days.

Recombinant mouse IL-1β (R&D Systems) was injected IP at 20 μg/kg in PBS at days 13, 17, 21, and 25 in the respective experiments.

Histopathology

Myocarditis severity was scored on hematoxylin/eosin stained heart sections using a semi-quantitative 0 to 4 scale (see the expanded Methods section in the Online Data Supplement at http://circres.ahajournals.org). Myocarditis severity was scored on hematoxylin/eosin stained heart sections using a semiquantitative 0 to 4 scale (see the expanded Methods section in the Online Data Supplement at http://circres.ahajournals.org). Histopathology

Heart inflammatory cells and splenocytes were isolated and processed as described.11 Cells were stained using fluorochrome-conjugated mouse-specific antibodies against CD45, CD11b, CD11c, CD4, Gr-1, F4/80, interferon-γ, IL-4, and IL-17 (all BD PharMingen). For intracellular cytokine stainings, cells were stimulated for 4 hour with 50 ng/mL phorbol myristate acetate and 500 ng/mL ionomycin in the presence of 10 μg/mL Brefeldin A (all Sigma-Aldrich). Heart infiltrates were identified as CD45+ cells from heart tissue suspensions gated on CD45/forward scatter plots as described.11

Flow Cytometry

Heart inflammatory cells and splenocytes were isolated and processed as described.11 Cells were stained using fluorochrome-conjugated mouse-specific antibodies against CD45, CD11b, CD11c, CD4, Gr-1, F4/80, interferon-γ, IL-4, and IL-17 (all BD PharMingen). For intracellular cytokine stainings, cells were stimulated for 4 hour with 50 ng/mL phorbol myristate acetate and 500 ng/mL ionomycin in the presence of 10 μg/mL Brefeldin A (all Sigma-Aldrich). Heart infiltrates were identified as CD45+ cells from heart tissue suspensions gated on CD45/forward scatter plots as described.11

Proliferation Assay

CD4+ T cells were purified using magnetic beads (CD4+ T cell isolation kit; Miltenyi Biotec). A total of 2×106 splenocytes or 5×104 CD4+ T cells cocultured with 106 irradiated (25 Gy) syngeneic splenocytes were restimulated for 48 hours in the presence of sequential dilutions of the MyHC-α or OVA peptide. Proliferation was assessed by measuring [3H]thymidine incorporation during the last 8 hours.

Real-Time RT-PCR

RNA isolation, mRNA transcription and PCR were performed as described.11,19 Oligonucleotides complementary to transcripts of the genes analyzed are listed in the Online Data Supplement. Transcript levels of gapdh were used as endogenous reference.

Isolation and Culture of Cardiac Fibroblasts

Cardiac fibroblasts were isolated and cultured as described20 with minor modifications (for details see Online Data Supplement).

Immunocytochemistry

Hearts were formalin-fixed and paraffin-embedded and cells were fixed with 4% paraformaldehyde. Immunostaining procedures were performed as described.19 Sections and cells were stained with rat anti-mouse-cardiac troponin I, rabbit anti-mouse fibronectin (Millipore), and goat anti-rabbit IgG Alexa Fluor 546 (Invitrogen). Nuclei were detected using DAPI (Pierce).

Echocardiography

Transesophageal echocardiography was performed using a 30 MHz probe and the V170 Ultrasound machine (VisualSonics). Detailed description is available in the Online Data Supplement.

Statistics

All data, except for the myocarditis severity scores, were considered as normally distributed and analyzed by unpaired, 2-tailed Student t test and for multiple comparisons, by 1-way or 2-way ANOVA followed by Bonferroni’s post test. Severity scores were analyzed by the 2-tailed Mann–Whitney U test and for multiple comparisons, by the Kruskal–Wallis 1-way analysis. Differences were considered as statistically significant for P<0.05.

Results

CFA Challenging Promotes Massive Cardiac Fibrosis in Mice With EAM

To study the potential role of TLR activation on the progression of acute myocarditis to inflammatory cardiomyopathy,
we induced acute myocarditis using heart-specific peptide (MyHC-α)-loaded bmDCs and boosted diseased mice with CFA, a nonspecific, strong TLR stimulant known to activate various TLR receptors. As illustrated in Figure 1, MyHC-α- but not OVA-loaded bmDC-immunized BALB/c mice developed myocarditis with CD45^+ inflammatory infiltrates in the myocardium and heart-specific autoreactive CD4^+ T-cell responses at day 10 after the first bmDC injection (Figure 1A through 1H). To specifically address the effects of CFA challenge on disease progression, we injected groups of MyHC-α- loaded bmDC-immunized mice with either CFA or PBS at days 10 and 17 and analyzed their hearts at day 34. CFA- but not sham-challenged mice developed massive fibrosis (Figure 1I and 1J). The fibrotic phenotype in CFA-challenged mice was consistently detected 120 days after bmDC immunization (data not shown). Importantly, CFA alone was not sufficient to induce any detectable fibrotic response at day 34 in OVA-loaded bmDC-immunized mice (Figure 1K).

CFA represents a nonspecific TLR stimulant. To clarify the role of specific TLRs in the progression of myocardial fibrosis, mice with acute myocarditis were boosted repetitively with selective TLR2- (ie, peptidoglycan), TLR4 (ie, lipopolysaccharide) or TLR9 (ie, Cpg) agonists, starting at day 10 postimmunization and the percentages of the fibrotic areas at day 34 were analyzed. As illustrated in Online Figure I, hearts of TLR stimulant- but not sham-treated mice showed significant fibrotic areas within their hearts. Compared with the massive fibrosis observed after CFA stimulation, the effects of repetitive TLR stimulant injections were modest albeit significantly higher compared with sham treatments. MyHC-α-Loaded bmDCs Induce Similar EAM Phenotypes in Wild-Type and MyD88^−/− Mice

Given the integrating role of the adaptor molecule MyD88 in various TLR pathways, we investigated the specific role of MyD88 signaling in the fibrotic response following acute myocarditis. As reported previously12,13 and illustrated in Figure 2, immunization with self-antigen–loaded wild-type bmDCs induce myocarditis of comparable severity in wild-type and MyD88^−/− mice at day 10 (Figure 2A and 2B). Importantly, we did not detect fibrotic areas in inflamed hearts of both mouse strains at this time point (Online Figure II). Furthermore, gene expression analysis showed no differences in the cardiac mRNA levels of proinflammatory cytokines, such as ifn-γ, il-4, il-17, il-33, ccl1, mcp-1, mip-1α, mip-1β, and mip-2 (Online Figure III), between wild-type and MyD88^−/− mice. To investigate whether the distributions of
the major heart-infiltrating cell subsets differ between wild-type and MyD88−/− mice, heart-infiltrating CD45+ cells were analyzed by flow cytometry. As shown in Figure 2C, we found no differences in the distribution of monocytes (CD11b), granulocytes (Gr-1), macrophages (F4/80), CD4+ T cells (CD4), and dendritic cells (CD11c) within the CD45+ heart-infiltrating cell subsets between wild-type and MyD88−/− mice at day 10 after bmDC immunization.

Next, we compared the autoreactive CD4+ T-cell responses in immunized wild-type and MyD88−/− mice. CD4+ T cells were isolated at day 10 from spleens of wild-type and MyD88−/− mice immunized with activated, MyHC-α-loaded bmDCs and stimulated with PBS or recombinant IL-1β (20 μg/kg) at days 13, 17, 21, and 25. Fibrotic areas were scored on Masson’s trichrome-stained heart sections at day 34. Individual values and the average for each group are shown. *P<0.05 by 1-way ANOVA (A) and by the 2-tailed Student t test (B).
Persistent Heart-Specific T-Cell Responses Do Not Explain Cardiac Fibrosis in Wild-Type Mice

So far, our findings suggest that both heart specific autoimmunity and MyD88 signaling are critical for cardiac fibrosis and heart failure progression. The question therefore arises whether these phenotypes reflect differences in autoreactive, heart-specific T-cell responses between MyD88−/− and wild-type mice during the late phases of disease. This, however, seems not to be the case, because most cardiac infiltrates resolved as early as at day 21 in both MyD88−/− and wild-type mice after CFA challenge (Figure 6A and Online Figure II). In wild-type mice, inflammatory cells were replaced by fibrotic tissue at this stage. Moreover, we found the same in vitro proliferation response of MyHC-α-specific CD4+ T cells isolated at late time points from spleens of MyD88−/− mice without versus wild-type mice with myocardial fibrosis (Figure 4B).

On the other hand, if bmDC-immunized mice were boosted not only with CFA but also with the antigenic MyHC-α peptide, we observed persistence of the myocarditis phenotype and marked inflammatory infiltrates without detectable fibrosis in both wild-type and MyD88−/− hearts up to day 21 (Figure 4A through 4C). Despite the ongoing recruitment of inflammatory cells to the hearts, MyD88−/− mice were protected from the development of massive cardiac fibrosis, ventricular dilation and heart failure at day 42 (Figure 4D, 4E, and 4G).

These findings indicate that the presence of an ongoing autoreactive T-cell response by itself does not automatically induce a fibrotic response and development of an end-stage heart failure phenotype.

IL-1β Mediates Myocardial Fibrosis

IL-1 is a key proinflammatory cytokine produced in response to TLR/MyD88 stimulation.22 Next, we specifically address the role of IL-1 signaling in tissue fibrosis. Accordingly, we challenged WT bmDC-immunized MyD88−/−, IL-1R−/−, and WT control mice with CFA, analyzed the expression of profibrotic genes in heart tissues, and assessed the extent of cardiac fibrosis on follow up.

Similar to MyD88−/− mice, but in contrast to wild-type animals, IL-1R−/− mice were completely protected from cardiac fibrosis at day 34 (Figure 3A). Real-time RT-PCR analysis revealed elevated expression of il-6 and collagen, as well as several genes known to be directly involved in tissue remodelling, such as mmp-8, mmp-9, timp-2, and timp-3 at day 21 (Figure 7) in wild-type but not in MyD88−/− and IL-1R−/− mice. These results correlate with the fibrotic phenotype observed at day 34 (Figure 3A). However, there was no correlation between the fibrotic phenotype and cardiac mRNA transcript levels of il-33, tgf-b1, tnf-a, sdf-1, mmp-2, and timp-1 (Figure 7). CFA challenge upregulated the expression of il-1β in wild-type and IL-1R−/− but not in MyD88−/− mice (Figure 7). This observation suggests that CFA triggered il-1β expression depends on MyD88 signaling in our model. Furthermore, our data imply that the MyD88/IL-1 axis is critical for the progressive tissue fibrosis, which parallels the transition of acute myocarditis to an end-stage heart failure. Accordingly, treatment of bmDC-immunized wild-type mice with IL-1β alone was sufficient to promote...
cardiac fibrosis and heart failure at day 34 (Figure 3B) but did not enhance cardiac inflammation at day 21 (Online Figure IV). In contrast, IL-1β treatments did not mediate cardiac fibrosis in MyD88−/− mice (data not shown).

MyD88/IL-1 Signaling in the BM-Derived Cellular Compartment Mediates Fibrosis

The BM represents the dynamic source of heart-infiltrating fibroblast progenitors in EAM.23 We therefore created chimeric mice to address the specific role of BM-derived cells in heart failure progression and tissue fibrosis. All chimeric mice were immunized with bmDCs, and for fibrosis development, animals were challenged with MyHC−CFA. In chimeric mice reconstituted with EGFP−BM, nearly all EGFP cells were CD45+, suggesting an inflammatory phenotype in the acute phase (d10, Figure 8A and 8B). At day 42, however, we found many EGFP-positive signals in the fibronectin-positive fibrotic tissue (Figure 8C and 8D), and in cardiac fibroblasts expanded from fibrotic hearts (Figure 8E). This implicates that BM-derived cells represent the major cellular substrate of cardiac fibrosis in our model. Next, we specifically addressed the role of MyD88/IL-1 signaling in the BM-derived cellular compartment for cardiac fibrosis and heart failure progression. As shown in Figure 8, wild-type mice reconstituted with MyD88−/− (MyD88−/−>WT) or IL-1R−/− (IL-1R−/−>WT) BM did not develop significant fibrosis, in contrast to control mice reconstituted with wild-type BM (WT->WT, Figure 8G). Instead, reconstitution of MyD88−/− or IL-1R−/− mice with wild-type BM (WT->MyD88−/− and WT->IL-1R−/−, respectively) was sufficient to induce severe fibrosis in our model (Figure 8G). Furthermore, increased heart/body weight ratios indicated ventricular dilatation and an end-stage heart failure pheno-
type (Figure 8H). Taken together, these data indicate that MyD88/IL-1 signaling in the BM-derived cellular compartment is critical for myocardial fibrosis and development of end-stage heart failure in inflammatory cardiomyopathy.

**Discussion**

Our data show for the first time the relevance of the MyD88/IL-1 axis in the progression of EAM to end-stage heart failure, thereby illustrating its critical role in cardiac fibrosis. Obviously, nonspecific activation of the IL-1/MyD88 axis is sufficient for heart failure progression once the heart experienced an acute inflammatory injury, and neither the presence of a specific pathogen nor the persistence of a dominant heart-specific T-cell response is required for the development of a typical end-stage heart failure phenotype.

Simply assessing myocarditis susceptibility of transgenic animals lacking defined components of the innate immune system does not allow a conclusion regarding their specific role at different time points during induction or progression of disease. Mice lacking MyD88, for example, are protected from both autoimmune and viral myocarditis caused by impaired virus replication and APCs function. However, the combined challenging with bmDC immunization and CFA offers an attractive strategy to overcome myocarditis resistance and to address the relevance of this specific genetic effect on heart failure progression. From clinical perspective, the identification of innate mediators promoting heart failure progression at later phases of the disease is of prime importance, because it may allow us to identify novel drug targets against heart failure.

Our data demonstrate that progression of myocarditis to cardiac fibrosis requires both prior cardiac inflammation and an additional nonspecific adjuvant effect promoting IL-1 release. TLRs serve as central targets for various immune-stimulating adjuvants and induce the activation of several proinflammatory genes, including il-1β. Clinical data suggest a role for TLR4 signaling in end-stage heart failure. Similarly, coxsackievirus represents a common infective agent promoting postinflammatory cardiomyopathy in Western countries that directly activates the immune system through TLRs. In animal experiments, TLR4-deficient mice indeed show reduced disease severity and fibrosis in a mouse model of coxsackievirusB3-induced myocarditis. In the commonly used EAM model, successful immunization with heart-specific self-antigen always requires costimulation with the
nonspecific adjuvant CFA. Our data indicate that CFA costimulation is not only required for the priming of autoreactive T cells but also promotes cardiac fibrosis independently of the autoreactive T-cell response. In addition, our findings may explain the marked fibrosis that is consistently observed in MyHC-α/CFA or coxsackievirus B3-immunized mice and the less pronounced fibrosis after immunization with self-antigen loaded bmDCs. 

Mechanistically, MyD88 signaling confers the activation of proinflammatory genes, including il-1β. Activation of the IL-1 type 1 receptor, on the other hand, also mediates its effects through the downstream adaptor molecule MyD88. In our model, delivery of the cytokine IL-1β alone was sufficient to promote cardiac fibrosis in the bmDC-immunized mice. IL-1R signaling regulated the expression of profibrotic genes including mmp-8 and -9, timp-2, and -3, and collagen. These findings are in line with observations that matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play an important role in myocarditis and myocardial remodelling. Furthermore, MyD88/IL-1 signaling has been shown to be essential for the development of fibrosis in bleomycin-induced lung injury, collagen-induced arthritis, and the healing processes after myocardial infarction and in the vascular remodeling.

Regarding the EAM model, IL-1 signaling apparently plays a dual role. On the one hand, it mediates activation of APCs and expansion of the self-pathogenic heart-specific CD4+ T-cell subset promoting myocarditis. On the other hand, it promotes fibrosis and heart failure progression at later stages of disease. This idea might explain why increased levels of il-1β transcripts in the myocardium show a linear correlation with cardiac function in coxsackievirus-mediated murine myocarditis and the observation that the local expression of an IL-1R antagonist improves survival in the same model.

In our model, the MyD88-dependent signaling appears to be the principle mediator of the development of postinflammatory heart failures. The combination of the CFA challenge with a boost of the self-peptide enhanced myocarditis severity in both wild-type and MyD88−/− mice but was insufficient to induce fibrosis the end-stage heart failure phenotype in MyD88−/− mice. This is important, given the fact that functional impairment and elevated levels of profibrotic mediators directly correlate with the extent of the inflammatory infiltrates. Obviously, acute cardiac inflammation is required but not sufficient for the development of myocardial fibrosis and heart failure progression.

Experiments with chimeric mice indicate a decisive role of the BM compartment as major substrate of cardiac fibrosis. Our observations clearly show that the heart failure phenotype depends on functional MyD88/IL-1 signaling in the BM-derived compartments only. Given the BM-derived cellular compartment represents the primary infiltrating cells into the heart during acute myocarditis, our data strengthen previous observations correlating the extent of heart failure to the level of cardiac infiltrates. Thus, our data clearly identify the BM-derived cellular compartment as a potential target for therapeutic interventions in end-stage heart failure.

Does that mouse system mirror human inflammatory dilated cardiomyopathy? Divergence of the postinflammatory fibrotic response found in our model is also observed in humans. Furthermore, several lines of evidence indicate that tissue damage promotes the release of endogenous TLR activators, such as heat-shock proteins. Consequently, ongoing TLR stimulation might contribute and promote the development of tissue fibrosis and heart failure. Clinical observations suggest that infections further enhance cardiac dysfunction in patients with severe heart failure. In addition upregulation of TLR as well as profibrotic genes are hallmarks of heart failure in humans. These facts suggest that indeed TLR signaling might enhance heart failure in inflammatory cardiomyopathy in humans as well.

Taken together, we found that activation of the MyD88/IL-1 axis is critical for tissue fibrosis and heart failure progression in inflammatory cardiomyopathy. Given findings from clinical long-term follow-up studies on humans with acute myocarditis demonstrating spontaneous improvement in 40% to 70% of patients and progression to dilated cardiomyopathy in 21% of patients, we can only speculate how the interplay of genetic and environmental factors orchestrate the individual susceptibility for the progression of myocarditis to end-stage heart failure. In this regard, our findings warrant a closer focus on the innate immune system in general and, in particular, the role of MyD88/IL-1 axis during this process. Finally, our findings suggest targeting the MyD88/IL-1 axis as a novel treatment strategy for patients with inflammatory dilated cardiomyopathy.

Acknowledgments
We thank Marta Bachmann and Heidi Bodmer for technical assistance and Prof Marc Y. Donath and Dr Galit Alter for critical reading of the manuscript.

Sources of Funding
We thank the Swiss Heart Foundation, the Olga Mayenfisch Foundation, the Hartmann Müller Foundation, and the Swiss Life Foundation for generous support. U.E. holds a Swiss National Foundation Professorship.

Disclosures
None.

References


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Circ Res. 2009;105:912-920; originally published online September 17, 2009;
doi: 10.1161/CIRCRESAHA.109.199802

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplementary on-line material

Supplementary methods

**Histopathology** Myocarditis severity was scored on hematoxylin-eosin stained heart sections using a semi-quantitative 0-4 scale: 0 – no inflammatory infiltrates; 1 – small foci of inflammatory cells between myocytes; 2 – larger foci of >100 inflammatory cells; 3 – >10% of a cross-section involved; 4 – >30% of a cross-section involved.

**Real-Time RT-PCR** The following oligonucleotides were used in this study:  
\[ ccl1: 5'-caaaagttggtccaaatca-3', 5'-ggggaaggtgctatcattca-3'; \]
\[ collagen I: 5'-gatgaagtgcaatgcaatgaa-3', 5'-ccctgactctcatcttctgtga-3'; \]
\[ gapdh: 5'-ctgcacccacactcttacctgta-3', 5'-ggcatggactgtgtgtatcag-3'; \]
\[ il-1β: 5'-caaccaacaagtattctctat-3', 5'-atccacacttccagcttca-3'; \]
\[ il-6: 5'-tctatgaacaacagccagag-3'; \]
\[ il-17: 5'-accgcaatgacacctgtgag-3', 5'-tcctccgcatttgaca-3'; \]
\[ il-33: 5'-gattggaagaaggtaggtgtga-3', 5'-ttgtgaagccgagaagagc-3'; \]
\[ mcp-1: 5'-tcactgaagccagcttcttct-3', 5'-gcaggccccagaagctga-3'; \]
\[ mip1-α: 5'-ttttggaaccgagcctttg-3', 5'-caggaaggtgggctggtc-3'; \]
\[ mip1-β: 5'-tgctcgtggctgccttct-3', 5'-caggaaggtgggctggtc-3'; \]
\[ mmp-2: 5'-ccctcaacgagcaagatctt-3', 5'-caggaaggtgggctggtc-3'; \]
\[ mmp-8: 5'-caacctattcttctctgtgct-3', 5'-tgctcgtttgctgcttct-3'; \]
\[ mmp-9: 5'-cctggcaactcagcagttc-3', 5'-tggaactcagcagttc-3'; \]
\[ sdf-1: 5'-gtggtaactttctctcctg-3', 5'-ttggaactcagcagttc-3'; \]
\[ timp-1: 5'-gcatggacattttctttcactgt-3', 5'-tctctagagccagcagttc-3'; \]
\[ timp-2: 5'-ttcgggaatgacatctggtggtt-3'; \]

1
3’, 5’-gggccgttagataaactcgat-3’; {	extit{ timp-3}: 5’-ggacgcagatgctctcaaat-3’, 5’-cggtaccagctgagcagctg-3’; {	extit{ tgf-β1}: 5’-gacgcagatgctctcaaat-3’, 5’-ggcgtagctgagcagctg-3’; \textit{tnf-α }5’-ccacccgctcaactcagtc-3’, 5’-ccctcacttgggtgtttgct-3’.

**Isolation and culture of cardiac fibroblasts** Hearts were perfused, isolated, transferred into Liberase Blendzyme 3 (Roche) enzymes, cut into small pieces and incubated 45 min at 37°C. Cell suspension and the remaining tissue were strained through 70µm cell strainer. Cardiomyocytes were removed by sedimentation by the gravity force for 8 min at 37°C. Cell suspension was plated in DMEM medium (Gibco) supplemented with 10% foetal calf serum and penicillin/streptomycin solution (Gibco). After overnight incubation, non-adherent cells were removed. Cells were cultured for 10-14 days, and medium was changed every 3-4 days.

**Echocardiography** Transthoracic echocardiography was performed using a 30 MHz probe and the Vevo 770 Ultrasound machine (VisualSonics). Mice were lightly anesthetized with 1-1.5% isoflurane, maintaining heart rate at 400-500 beats per minute. Diastolic and systolic internal ventricular septum (IVS;d and IVS;s), diastolic and systolic left ventricular free posterior wall thickness (LVPW;d and LVPW;s), and left ventricular internal end-diastolic and end-systolic chamber (LVID;d and LVID;s) dimensions were measured. The measurements were taken in three separate M-mode images and averaged. Left ventricular fractional shortening (%FS) and ejection fraction (%EF) were also calculated. %EF was derived from the formula of (LV vol;d – LV vol;s)/ LV vol;d*100. Pulse doppler images were collected with the apical four-chamber
view to record the mitral doppler flow spectra. The doppler sample volume was placed at the level of the left ventricle and moved toward the left ventricular outflow tract to intersect with both the mitral inflow and the left ventricular outflow in the same recording. Pulse wave doppler tissue imaging (DTI) was also performed on mice by activating the DTI function in Vevo 770 echocardiography. Sample volume was measured at the septal side of the mitral annulus velocity and the ratio E’ to A’ was determined.
Supplementary figure legends

Supplementary Figure 1

Various TLR stimulants promote post-inflammatory fibrosis.

Wild-type (WT) mice were immunized with MyHC-a-loaded bmDCs and challenged with selective stimuli for TLR2 [peptidoglycan (PDG)], TLR4 [lipopolysaccharides (LPS)] or TLR9 (CpG) for 14 days. Fibrotic areas were scored on Masson’s trichrome stained heart sections at day 34. Individual values and the average for each group are shown. *p<0.05 by one-way ANOVA.

Supplementary Figure 2

WT but not MyD88−/− mice develop post-inflammatory fibrosis.

Representative heart tissue sections stained with Masson’s trichrome of WT and MyD88−/− mice immunized with MyHC-a-loaded bmDCs and challenged with CFA at different stages. Scale bar = 100µm.

Supplementary Figure 3

Expression of pro-inflammatory genes in WT and MyD88−/− mice.

WT and MyD88−/− mice were immunized with MyHC-a-loaded bmDCs and heart tissues were assessed with Real-Time RT-PCR for the expression of pro-inflammatory genes at day 0 and 10. Bars represent mean ± SD from at least 6 individual mice. p>0.05 (n.s.) by two-way ANOVA. n.d. = not detected.

Supplementary Figure 4
Stimulation with IL-1β does not affect resolution of inflammation

Wild-type mice were immunized with MyHC-α-loaded bmDCs, challenged with PBS or recombinant IL-1β (20 µg/kg) at days 13 and 17, and analyzed for myocarditis development at day 21. Myocarditis severity was scored on heart sections stained with hematoxylin-eosin. Individual values are displayed. p>0.05 (n.s.) by the two-tailed Mann-Whitney U test.
Supplementary figure 3
Supplementary figure 4