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Abstract

RATIONALE: The myeloid differentiation factor (MyD)88/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 alpha-myosin heavy chain peptide (MyHC-alpha)-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-alpha/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.
MyD88/IL-1 signalling controls cardiac fibrosis and heart failure progression in inflammatory dilated cardiomyopathy

Running title: MyD88/IL-1 promotes heart failure

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Abstract

Rationale: The MyD88/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 alpha-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 signalling on bone marrow-derived cells was critical for development of cardiac fibrosis during progression to heart failure.

Conclusion: Our findings indicate a critical role of MyD88/IL-1 signalling in the bone marrow compartment in post-inflammatory cardiac fibrosis and heart failure, and point to novel therapeutic strategies against inflammatory cardiomyopathy.

Key words: autoimmune myocarditis, heart failure, fibrosis, innate immunity

Non-standard Abbreviations and Acronyms: APC - antigen presenting cell, BM - bone marrow, bmDC - bone marrow-derived dendritic cell, CFA - complete Freund’s adjuvant, EAM - experimental autoimmune myocarditis, MyHC-α - alpha-myosin heavy chain, OVA - ovalbumine, TLR - Toll-like receptor
Introduction

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\(^2,3\). 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\(^4,7\). 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 CD\(^4^+\) T cells\(^7,11\). We found that cardiac damage of any cause, together with non-specific 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 alpha-myosin heavy chain (MyHC-\(\alpha\)) peptide induce acute CD\(^4^+\) T cell mediated myocarditis in susceptible mice\(^12,13\). IL-1 receptor and most of the TLRs share the common downstream signalling adaptor molecule MyD\(88\)\(^14,15\). MyD\(88\)- and IL-1R-signalling on APCs are critical for the induction of heart-specific T cell responses and EAM. However, adoptive transfer of wild-type bmDC with intact IL-1R/MyD\(88\) signalling restores myocarditis susceptibility of MyD\(88^-\) 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\textsuperscript{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 signalling 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.
Materials and Methods

**Mice** BALB/c (n=125), MyD88\(^{-/-}\) (n=60), IL-1R\(^{-/-}\) (n=17) mice (maintained on a BALB/c background as described previously\(^{13,16}\)) and C57BL/6-EGFP transgenic mice (EGFP under the control of the β-actin promoter) backcrossed into BALB/c background for >10 generations (hereafter referred to as BALB/c-EGFP, n=8) were used at 6-8 week of age. Animal experiments were performed in accordance with the Swiss federal law and were approved by the local authorities.

**Chimeric mice** 6-8 week old mice were lethally irradiated with two doses of 6.5 Gy as described\(^{11}\) and transplanted with total of 2x10\(^7\) 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-α\(_{614-634}\) (Ac-SLKLMATLFSTYASAD-OH) or ovalbumine (OVA\(_{323-339}\)) peptide, activated with 0.1 µg/mL lipopolysaccharide and 5 µg/mL anti-CD40 prior to intraperitoneal injection of 5x10\(^5\) cells/mouse at days 0, 3 and 5. In the respective experiments, mice received additional subcutaneous injections of 200 µL PBS or CFA emulsified with PBS (1:1) with or without 100 µg MyHC-α at days 10 and 17. In some experiments, bmDC immunized mice were repetitively, every second day, injected with 1 µg/ml lipopolysaccharide (Escherichia coli 0111:B4; Sigma), 10 µM CpG-ODN or 10 µg/ml peptidoglycan (Staphylococcus aureus; Fluka) for 14 days.
Recombinant mouse IL-1β (R&D Systems) was injected intraperitoneally 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 0-4 scale (see Supplementary on-line material). Masson’s trichrome staining was used to detect fibrosis. The degree of fibrosis was calculated as % of the fibrotic area in relation to the total heart area using analySIS FIVE software (Olympus).

**Flow cytometry** Heart inflammatory cells and splenocytes were isolated and processed as described\textsuperscript{11,19}. Cells were stained using fluorochrome-conjugated mouse-specific antibodies against CD45, CD11b, CD11c, CD4, Gr-1, F4/80, IFN-γ, IL-4 and IL-17 (all BD Pharmingen). For intracellular cytokine stainings, cells were stimulated for 4 h 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\textsuperscript{+} cells from heart tissue suspensions gated on CD45/forward scatter plots as described\textsuperscript{11}.

**Proliferation assay** CD4\textsuperscript{+} T cells were purified using magnetic beads (CD4\textsuperscript{+} T cell isolation kit; Miltenyi Biotec). A total of 2x10\textsuperscript{5} splenocytes or 5x10\textsuperscript{4} CD4\textsuperscript{+} T cells co-cultured with 10\textsuperscript{5} irradiated (25 Gy) syngeneic splenocytes were re-stimulated for 48 h in the presence of sequential dilutions of the MyHC-α or OVA peptide. Proliferation was assessed by measuring [\textsuperscript{3}H]-thymidine incorporation during the last 8 hours.
**Real-Time RT-PCR** RNA isolation, mRNA transcription and PCR were performed as described\textsuperscript{11,19}. Oligonucleotides complementary to transcripts of the genes analyzed are listed in the supplementary on-line material. Transcript levels of *gapdh* were used as endogenous reference.

**Isolation and culture of cardiac fibroblasts** Cardiac fibroblasts were isolated and cultured as described\textsuperscript{20} with minor modifications (for details see supplementary on-line material).

**Immunocytochemistry** Hearts were formalin-fixed and paraffin-embedded and cells were fixed with 4\% paraformaldehyde. Immunostaining procedures were performed as described\textsuperscript{19}. Sections and cells were stained with rat anti-mouse CD45 (BD Pharmingen), rabbit anti-mouse fibronectin (Millipore) and goat anti-rabbit IgG Alexa Fluor 546 (Invitrogen). Nuclei were detected using DAPI (Pierce).

**Echocardiography** Transthoracic echocardiography was performed using a 30 MHz probe and the Vevo 770 Ultrasound machine (VisualSonics). Detailed description is available in the supplementary on-line material.

**Statistics** All data, except for the myocarditis severity scores, were considered as normally distributed and analyzed by unpaired, two-tailed Student’s *t*-test, and for multiple comparisons by one-way or two-way ANOVA followed by Bonferroni’s post-test. Severity scores were analyzed by the two-tailed Mann-Whitney *U* test, and for
multiple comparisons by the Kruskal-Wallis one-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 non-specific, 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 (Figs. 1A-H). 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 (Figs. 1I,J). 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 bmDCs immunized mice (Fig. 1K).

CFA represents a non-specific 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- (i.e. peptidoglycan), TLR4- (i.e. lipopolysaccharide) or TLR9-agonists (i.e. CpG), starting at day 10 post-immunization and the percentages of the fibrotic areas at day 34 were analyzed. As illustrated in Suppl. Fig. 1, 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 bmDC 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 signalling in the fibrotic response following acute myocarditis. As reported previously 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 (Figs. 2A,B). Importantly, we did not detect fibrotic areas in inflamed hearts of both mouse strains at this time point (Suppl. Fig. 2). Furthermore, gene expression analysis showed no differences in the cardiac mRNA levels of pro-inflammatory cytokines such as, *ifn-γ, il-4, il-17, il-33, ccl1, mcp-1, mip-1α, mip-1β and mip-2* (Suppl. Fig. 3) 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 Fig. 2C, we found no differences in the distribution of monocytes (CD11b), granulocytes (Gr-1), macrophages (F4/80), CD4+ T cells (CD4), and DCs (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 re-stimulated
in the presence of APCs and sequential dilutions of the MyHC-α peptide. As demonstrated, both wild-type and MyD88−/− CD4+ T cells showed similar proliferation responses upon MyHC-α peptide stimulation (Fig. 2D). In addition, we found no differences in the percentages of IFNγ-, IL-4- and IL-17-producing CD4+ T cell subsets between diseased wild-type and MyD88−/− mice (Fig. 2E). Thus, immunization with bmDCs induced the same myocarditis phenotype regarding prevalence, severity of inflammation, distribution of heart infiltrating cells, and CD4+ T cell responses in wild-type and MyD88−/− mice.

**MyD88−/− mice are protected from fibrosis and heart failure**

As illustrated above, CFA greatly accelerates cardiac fibrosis in the EAM model. To define the role of MyD88 signalling in the fibrotic response and its effects on the development of an end stage heart failure phenotype, wild-type and MyD88−/− mice were immunized with MyHC-α loaded, activated WT bmDCs and challenged with either CFA or MyHC/CFA at days 10 and 17. Wild-type, but not MyD88−/− mice developed severe cardiac fibrosis at day 34 (Fig. 3A) and 42 (Fig. 4H) respectively. Development of cardiac fibrosis was associated with massive dilation of the left ventricle, increased heart/body weight ratios (Figs. 4D-F), and severely impaired cardiac functions in wild-type, but not MyD88−/− mice (Fig. 5A,B) reflecting an end-stage heart failure phenotype. Mirroring the profound fibrotic response, wild-type, but not MyD88−/− mice showed marked diastolic dysfunction, i.e. a restrictive left ventricular filling pattern reflected by an increase in the E/A inflow pattern (Figs. 5C-E).
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 signalling are critical for cardiac fibrosis and heart failure progression. The question therefore arises, whether these phenotypes reflect differences in auto-reactive, heart-specific T cells 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 (Fig. 6A and Suppl. Fig. 2). 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 vs. wild-type mice with myocardial fibrosis (Fig. 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 (Fig. 4A-C). 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 (Figs. 4D,E,G).

These findings indicate that the presence of an ongoing auto-reactive 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 pro-inflammatory cytokine produced in response to TLR/MyD88 stimulation\textsuperscript{22}. Next, we specifically address the role of IL-1 signalling in tissue fibrosis. Accordingly, we challenged WT bmDC immunized MyD88\textsuperscript{+/--}, IL-1R\textsuperscript{+/--}, and WT control mice with CFA, analyzed the expression of pro-fibrotic genes in heart tissues, and assessed the extent cardiac fibrosis on follow up.

Similar to MyD88\textsuperscript{+/--} mice, but in contrast to wild-type animals, IL-1R\textsuperscript{+/--} mice were completely protected from cardiac fibrosis at day 34 (Fig. 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 (Fig. 7) in wild-type, but not in MyD88\textsuperscript{+/--} and IL-1R\textsuperscript{+/--} mice. These results correlate with the fibrotic phenotype observed at day 34 (Fig. 3A). However, there was no correlation between the fibrotic phenotype and cardiac mRNA transcript levels of il-33, tgf-\(\beta\)1, tnf-\(\alpha\), sdf-1, mmp-2 and timp-1 (Fig. 7). CFA challenge up-regulated the expression of il-1\(\beta\) in wild-type and IL-1R\textsuperscript{+/--} but not in MyD88\textsuperscript{+/--} mice (Fig. 7). This observation suggests that CFA triggered il-1\(\beta\) expression depends on MyD88 signalling 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-1\(\beta\) alone was sufficient to promote cardiac fibrosis and heart failure at day 34 (Fig. 3B), but did not enhance cardiac inflammation at day 21 (Suppl. Fig. 4). In contrast, IL-1\(\beta\) treatments did not mediate cardiac fibrosis in MyD88\textsuperscript{+/--} or IL-1R1\textsuperscript{+/--} mice (not shown).

\textit{MyD88/IL-1 signalling in the BM-derived cellular compartment mediates fibrosis}
The BM represents the dynamic source of heart-infiltrating fibroblast progenitors in EAM\textsuperscript{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\textsuperscript{+} BM, nearly all EGFP\textsuperscript{+} cells were CD45\textsuperscript{+} suggesting an inflammatory phenotype in the acute phase (d10, Figs. 8A,B). At day 42, however, we found many EGFP-positive signals in the fibronectin-positive fibrotic tissue (Figs. 8C,D), and in cardiac fibroblasts expanded from fibrotic hearts (Fig. 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 signalling in the BM-derived cellular compartment for cardiac fibrosis and heart failure progression. As shown in Figure 8, wild-type mice reconstituted with MyD88\textsuperscript{+/-} (MyD88\textsuperscript{+/-}->WT) or IL-1R\textsuperscript{+/-} (IL-1R\textsuperscript{+/-}->WT) BM did not develop significant fibrosis, in contrast to control mice reconstituted with wild-type BM (WT->WT, Fig. 8G). Instead, reconstitution of MyD88\textsuperscript{+/-} or IL-1R\textsuperscript{+/-} mice with wild-type BM (WT->MyD88\textsuperscript{+/-} and WT->IL-1R\textsuperscript{+/-} respectively) was sufficient to induce severe fibrosis in our model (Fig. 8G). Furthermore, increased heart/body weight ratios indicated ventricular dilatation and an end stage heart failure phenotype (Fig. 8H). Taken together, these data indicate that MyD88/IL-1 signalling 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, non-specific 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 are 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 due to impaired virus replication and APCs function\textsuperscript{13,24}. 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, as 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 non-specific adjuvant effect promoting IL-1 release. TLRs serve as central targets for various immune stimulating adjuvants\textsuperscript{21} and induce the activation of several pro-inflammatory genes, including $il-1\beta$. Clinical data suggest a role for TLR4 signalling in end stage heart failure. Similarly,
Coxsackievirus represents a common infective agent promoting post-inflammatory 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 coxsackievirus B3-induced myocarditis\textsuperscript{25}. In the commonly used EAM model, successful immunization with heart-specific self-antigen always requires co-stimulation with the non-specific adjuvant CFA. Our data indicate that CFA co-stimulation 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-\(\alpha\)/CFA or coxsackievirus B3 immunized mice and the less pronounced fibrosis after immunization with self-antigen loaded bmDCs\textsuperscript{12,26,27}.

Mechanistically, MyD88 signalling confers the activation of pro-inflammatory genes, including \textit{il-1}\(\beta\). 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-1\(\beta\) alone was sufficient to promote cardiac fibrosis in the bmDC-immunized mice. IL-1R signalling regulated the expression of pro-fibrotic genes including \textit{mmp-8} and -9, \textit{timp-2}, -3 and -4 and \textit{collagen}. These findings are in line with observations that MMPs and TIMPs play an important role in myocarditis and myocardial remodelling\textsuperscript{27,29}. In addition, Bujak \textit{et al.} reported that IL-1 signalling on cardiac fibroblasts regulates MMPs and TIMPs expression\textsuperscript{30}. Furthermore, MyD88/IL-1 signalling has been shown to be essential for the development of fibrosis in bleomycin-induced lung injury\textsuperscript{31}, collagen-induced arthritis\textsuperscript{32}, as well as in the healing processes after myocardial infarction\textsuperscript{30} and in the vascular remodelling\textsuperscript{33}. 
Regarding the EAM model, IL-1 signalling 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\textsuperscript{16,34}, 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-1\textbeta transcripts in the myocardium show a linear correlation with cardiac function in coxsackievirus-mediated murine myocarditis\textsuperscript{35}, and the observation that the local expression of an IL-1R antagonist improves survival in the same model\textsuperscript{36}.

In our model, the MyD88-dependent signalling appears to be the principle mediator of the development of post-inflammatory heart failures. The combination of the CFA challenge with a boost of the self-peptide enhanced myocarditis severity in both wild-type and MyD88\textsuperscript{-/-} mice, but was insufficient to induce fibrosis the end stage heart failure phenotype in MyD88\textsuperscript{-/-} mice. This is important, given the fact that functional impairment and elevated levels of pro-fibrotic mediators directly correlate with the extent of the inflammatory infiltrates\textsuperscript{17,27,28,37,38}. 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 signalling 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 strengthens 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 post-inflammatory 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, for example. 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 up-regulation of TLR as well as pro-fibrotic genes are hallmarks of heart failure in humans\textsuperscript{39,40}. These facts suggest, that indeed, TLR signalling 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 40-70\% of patients and progression to dilated cardiomyopathy in 21\% of patients\textsuperscript{41}, 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\textsuperscript{42}. 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.
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Disclosures

none
References


Figure legends

Figure 1

**CFA promotes myocardial fibrosis after myocarditis.**

MyHC-α-loaded bmDCs induce myocarditis (B) with massive infiltration of CD45⁺ cells (E) at day 10, whereas mice injected with PBS (A,D) or with OVA-loaded bmDCs (C,F) showed no sign of cardiac inflammation and (A,C) and very few CD45⁺ cells in the myocardium (D,F). To assess antigen-specific T cell responses, CD4⁺ T cells were isolated from mice immunized with MyHC-α- (open circles) or OVA-loaded (filled circles) bmDCs at day 10 and in vitro re-stimulated with MyHC-α (G) or OVA (H) peptides on irradiated syngeneic splenocytes for 48 hours. Data represent mean ± SD of triplicates from one out of three independent experiments.

Heart tissue sections were stained with Masson’s trichrome and analyzed for fibrosis at day 34. Fibrotic areas were observed in mice immunized with MyHC-α-loaded bmDCs boosted with CFA (I), but not in PBS treated animals (J). CFA boosting of mice immunized with OVA-loaded bmDCs failed to induce fibrosis (K). *p<0.05 by two-way ANOVA. Scale bar = 100μm.

Figure 2

**Characteristics of autoimmune myocarditis in MyD88⁻⁻ mice.**

Wild-type MyHC-α-loaded bmDCs induce a similar degree of myocarditis in wild-type (WT, A) and MyD88⁻⁻ (B) mice. Analysis of CD11b, Gr-1, F4/80, CD4 and CD11c expressing cells, gated on CD45⁺ heart infiltrates isolated from wild-type (solid bars) and MyD88⁻⁻ (open bars) mice at day 10 (C). Bars represent mean ± SD from at least 5 mice.
CD4\(^+\) T cells were isolated from spleens of wild-type (filled circles) and MyD88\(^{-/-}\) (open circles) mice at day 10 and *in vitro* re-stimulated with MyHC-\(\alpha\) peptide on irradiated syngeneic splenocytes (D). Data represent mean ± SD of triplicate samples from one representative out of four independent experiments.

Cytokine production by splenic CD4\(^+\) T cells of WT (solid bars) and MyD88\(^{-/-}\) (open bars) mice at day 0 and 10 (E). Bars represent mean ± SD from 3 mice.

\(p>0.05\) (n.s.) by the two-tailed Student’s t-test (C) and by two-way ANOVA (D,E). Scale bar = 100\(\mu\)m.

**Figure 3**

**MyD88 and IL-1 mediate fibrosis development.**

(A) Wild-type (WT), MyD88\(^{-/-}\) and IL-1R\(^{-/-}\) mice were immunized with MyHC-\(\alpha\)-loaded bmDCs and challenged with PBS or CFA at day 10 and 17.

(B) Wild-type mice were immunized with MyHC-\(\alpha\)-loaded bmDCs and stimulated with PBS or recombinant IL-1\(\beta\) (20 \(\mu\)g/kg) at day 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 one-way ANOVA (A) and by the two-tailed Student’s t-test (B).

**Figure 4**

**MyD88\(^{-/-}\) mice develop chronic myocarditis, but are protected from post-inflammatory heart failure**
Masson’s trichrome staining of heart sections of wild-type (WT, A,D) and MyD88<sup>−/−</sup> (B,E) mice immunized with MyHC-α-loaded bmDCs and challenged with MyHC-α/CFA at day 10 and 17. Both strains showed massive inflammation without detectable collagen deposition at day 21 (A,B). Myocarditis severity at day 21 was scored on heart sections stained with hematoxylin-eosin. Individual values and the median for each group are shown (C). However, wild-type but not MyD88<sup>−/−</sup> mice showed heart dilatation and massive fibrosis at day 42 (D,E). Heart/body weight (HW/BW) ratios (F) and fibrotic areas (G) scored on Masson’s trichrome stained heart sections at day 42 are presented for wild-type and MyD88<sup>−/−</sup> mice as individual values with the average for each group.

p>0.05 (n.s.) by the two-tailed Mann-Whitney U test (C), *p<0.05 by the two-tailed Student’s t-test (F,G). Scale bar = 100µm.

**Figure 5**

**MyD88<sup>−/−</sup> mice are protected from impaired heart function.**

Echocardiography was performed on wild-type (WT, solid bars) and MyD88<sup>−/−</sup> (open bars) mice at day 0, and 42 days after disease induction with bmDCs and challenge with MyHC/CFA. Percent of fractional shortening (%FS, A) and percent of ejection fraction (%EF, B) represent measurements for cardiac contractility. E/A ratios (C) reflecting diastolic doppler tracings of mitral flow, and E`/A` ratios (D) reflecting mitral annulus velocity patterns represent readouts for diastolic function. E displays early, and A late diastolic inflow (mm/s). E` and A` correspond to early and late mitral annulus velocity (mm/s), respectively. In contrast to MyD88<sup>−/−</sup> mice, diseased wild-type mice show inversion of the E`/A` ratio and marked increase of the E/A ratio. These patterns indicate
impaired, restrictive diastolic filling. Data from one out of three experiments with similar results are shown. Data represent mean ± SD from 6 wild-type vs. 6 MyD88−/− mice. *p<0.05 by two-way ANOVA.

(E) Doppler tracing of diastolic mitral inflow. The left panel shows a normal tracing pattern in a wild-type mouse at day 0. The right panel exemplifies a restrictive pattern with a high E/A ratio in the same mouse with end stage heart failure 42 days after disease induction.

Figure 6

Resolution of inflammation is minimally depended on MyD88.

Wild-type (WT) and MyD88−/− mice were immunized with MyHC-α-loaded bmDCs, challenged with PBS or CFA, and analyzed at day 21.

(A) Myocarditis severity was scored on heart sections stained with hematoxylin-eosin. Individual values and the median for each group are shown. p>0.05 by the Kruskal-Wallis one-way analysis.

(B) Splenocytes were isolated and re-stimulated in vitro with MyHC-α peptide for 48 hours. The data represent mean ± SD of triplicate culture wells from one representative out of two independent experiments. p>0.05 by two-way ANOVA.

Figure 7

Expression of profibrotic genes is MyD88- and IL-1-dependent.

Wild-type (WT), MyD88−/− and IL-1R−/− mice were immunized with MyHC-α-loaded bmDCs and challenged with PBS or CFA. Real-Time RT-PCR was performed on heart
tissue lysates to assess the expression of pro-fibrotic genes at day 21. Bars represent mean ± SD from at least 5 mice. *p<0.05 by one-way ANOVA.

Figure 8

Post-inflammatory heart pathology requires MyD88/IL-1 signalling in the BM-derived cell compartment.

All chimeric mice were immunized with MyHC-α-loaded bmDCs and challenged with MyHC/CFA 6 weeks after BM reconstitution.

(A-D) Analysis of chimeric mice reconstituted with EGFP+ BM. Flow cytometry analysis shows heart-infiltrating EGFP+ cells (A) expressing CD45 (B) at day 10 after immunization. Immunofluorescence analysis shows EGFP expression (D) in fibronectin-positive (C) fibrotic cardiac tissue at day 42.

(E,F) Fibronectin-producing cardiac fibroblasts expanded from fibrotic hearts (d42) of the chimeric mice reconstituted with EGFP+ BM (E), and of wild-type controls (F).

(G,H) Heart tissue sections of chimeric mice reconstituted with BM of the indicated donors were stained with Masson’s trichrome and analyzed for fibrosis at day 42 after immunization. Fibrotic areas (G) and heart/body weight (HW/BW) ratios (H) are presented as individual values with the average for each group. Scale bar = 20µm, WT – wild-type, *p<0.05 by one-way ANOVA.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8