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Cardiac inflammation in genetic dilated cardiomyopathy caused by *MYBPC3* mutation

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ABSTRACT

Cardiomyopathies are a leading cause of heart failure and are often caused by mutations in sarcomeric genes, resulting in contractile dysfunction and cellular damage. This may stimulate the production of a robust proinflammatory response. To determine whether myocardial inflammation is associated with cardiac dysfunction in dilated cardiomyopathy (DCM) caused by *MYBPC3* mutation, we used the well-characterized cMyBP-C$^{(t/t)}$ mouse model of DCM at 3 months of age. Compared to wild type (WT) mice, DCM mice exhibited significantly decreased fractional shortening (36.4 ± 2% vs. 15.5 ± 1.0%, $p < 0.0001$) and significantly increased spleen weight (5.3 ± 0.3 vs. 7.2 ± 0.4 mg/mm, $p = 0.002$). Intriguingly, flow cytometry analysis revealed a significant increase in total ($CD45^+CD11b^+Ly6C^-MHCII^+F480^+$) macrophages (6.5 ± 1.4% vs. 14.8 ± 1.4%, $p = 0.002$) and classically activated ($CD45^+CD11b^+Ly6C^-MHCII^+F480^+CD206^-$) proinflammatory (M1) macrophages (3.4 ± 0.8% vs. 10.3 ± 1.2%, $p = 0.0009$) in DCM hearts as compared with WT hearts. These results were further confirmed by immunofluorescence analysis of heart tissue sections. Splenic red pulp ($CD11b^+Ly6C^+MHCII^{low}F480^{hi}$) macrophages were significantly elevated (1.3 ± 0.1% vs. 2.4 ± 0.1%, $p = 0.0001$) in DCM compared to WT animals. Serum cytokine analysis in DCM animals exhibited a significant increase (0.65 ± 0.2 vs. 2.175 ± 0.5 pg/mL, $p = 0.02$) in interleukin (IL)-6 compared to WT animals. Furthermore, RNA-seq analysis revealed the upregulation of inflammatory pathways in the DCM hearts. Together, these data indicate a robust proinflammatory response in DCM hearts, likely in response to cellular damage triggered by *MYBPC3* mutation and resultant contractile dysfunction.

1. Introduction

Heart failure (HF), the final common pathway of many cardiovascular diseases, is a global health problem that afflicts an estimated 5.8 million Americans [1,2] and 30–50 million patients worldwide [3]. Cardiomyopathies are a leading cause of HF and are defined by a pathologically abnormal myocardium [4]. They are classified into four major categories: dilated (DCM), hypertrophic (HCM), restrictive (RCM), and arrhythmogenic right ventricular cardiomyopathy (ARVC) [4]. Genetic mutations underlie a significant proportion of cardiomyopathies [4]. For example, 75% of inherited HCMs are caused by mutations in the genes encoding myosin heavy chain (*MYH7*) and cardiac myosin binding protein-C (cMyBP-C, *MYBPC3*) [4]. Compared to HCM, DCM is a more heterogeneous disease, the causes of which may be idiopathic, familial/genetic, viral, and/or immune [5]. Intriguingly, an estimated 35%–40% of genetic DCMs are thought to arise from mutations in sarcomeric genes [4]. In genetic forms of cardiomyopathy, sarcomere protein mutations may generate severe mechanical stress as a result of contractile deficiencies that are unable to meet the cellular demands required for normal contractile function [1]. This deterioration in contractile function correlates with exacerbated cardiac myocyte damage or death, which, in turn, may trigger myocardial inflammation, further aggravating the progression of cardiomyopathy [1,6].

Abbreviations: ARVCM, arrhythmogenic right ventricular cardiomyopathy; cMyBP-C, cardiac myosin binding protein-C; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; HF, heart failure; IFN, interferon; IL, interleukin; LV, left ventricle; MCP, monocyte chemoattractant protein; RCM, restrictive cardiomyopathy; TNF, tumor necrosis factor.

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Inflammation has been recognized as a hallmark of HF [7] with levels of circulating and cardiac proinflammatory cytokines indicating HF severity in humans [8], as well as contributing to many of the pathological responses during HF in animals [7,9,10]. Based on this inflammatory response to damaged myocardium, researchers formulated the cytokine hypothesis, which holds that proinflammatory cytokines are produced by the damaged myocardium during chronic HF and that this inflammatory response promotes monocyte activation and the further production of cytokines, thus augmenting cardiac dysfunction [11]. However, to date, no large-scale therapeutic strategies targeting proinflammatory protein mediators in HF have been successfully translated to clinical practice, indicating a profound underappreciation of the role of inflammation in HF [7]. As such, we have recently suggested that attention be turned towards modulation of the underlying inflammatory cellular networks, including monocyte/macrophage populations, responsible for cytokine production, as well as regulation and generation of immune responses [7]. Indeed, macrophages and monocytes play a central role in inflammation and innate immunity [7,12]. The mammalian heart has been shown to contain a population of resident macrophages that proliferate following myocardial infarction and hemodynamic stress that signals further monocyte recruitment to the heart, contributing to myocardial interstitial fibrosis and adverse cardiac remodeling [12]. However, in cardiomyopathies caused by sarcomeric protein mutation, cardiac inflammation, as it manifests relative to contractile dysfunction and cardiac remodeling, has not been elucidated.

We recently reported the use of a well-characterized mouse model of genetic DCM harboring a homozygous knock-in mutation in the *MYBPC3* gene that translates into a truncated variant of cMyBP-C [13–16]. Using this mouse model (cMyBP-C$^{(t/t)}$), we demonstrated that oxidative stress, which is significantly elevated in genetically induced cardiomyopathy, correlates with the level of contractile dysfunction, cellular damage and cardiac remodeling [1]. In the current study, we use the cMyBP-C$^{(t/t)}$ mouse model to test the hypothesis that myocardial inflammation associates with cardiac dysfunction in dilated cardiomyopathy caused by *MYBPC3* mutation. Our data indicate a substantial elevation in the proinflammatory macrophages in cMyBP-C$^{(t/t)}$ DCM hearts and that such elevation is likely a response to cellular damage triggered by *MYBPC3* mutation and the resultant contractile dysfunction.

2. Materials and methods

2.1. Mouse model of DCM

In this study, we used a 3-month-old knock-in mouse model of DCM [17], in which a homozygous mutation in *MYBPC3* causes a C′-modified cMyBP-C (cMyBP-C$^{(t/t)}$) that does not incorporate into sarcomeres, resulting in a cMyBP-C null heart [16,17], and age-matched wild type (WT) mice (FVB/N strain). Male animals were used for these studies. All animal protocols were approved by the Institutional Animal Care and Use Committee at Loyola University Chicago (LU# 205109) and were implemented in accordance with the guidelines listed in the *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health. Prior to organ harvest, animals were euthanized in a carbon dioxide (CO_2) chamber by slow flow of CO_2 (10–30% of chamber volume per minute), followed by continued exposure for 15–30 min after breathing had stopped.

2.2. In vivo cardiac function by noninvasive echocardiography

Echocardiography using a Vevo 2100 (Fujifilm, Visual Sonics, Inc., Toronto, Canada) and an MS-550D 22–55 MHz transducer was performed to assess cardiac function in WT and DCM mice anesthetized with 1.5%–2% isoflurane in 100% oxygen via inhalation. Left ventricular internal diameter (LVID), wall thickness, and contractile function were measured using short-axis M-mode imaging and the Visual Sonics Vevo 2100 analysis package as described previously [1].

2.3. Histopathological analysis of splenic architecture

To evaluate general spleen histology, spleens were isolated from WT and DCM mice and then formalin-fixed and embedded in paraffin. Spleens were sectioned at 5-μm thickness, deparaffinized, rehydrated, and stained with Masson's trichrome as previously described [7].

2.4. Isolation of mononuclear cells from the heart

Single mononuclear cells were isolated from WT and DCM hearts as previously described [7]. Briefly, mouse hearts were completely excised; following removal of fatty tissue and aorta, they were placed in heparinized saline. Hearts were then minced into fine pieces, and blood was removed by repeated wash with saline. The tissue was digested in 10 mL of digestion buffer for 50 min at 37 °C with constant shaking. Released cells were separated from solid tissue by filtration through a 70-μm nylon cell-strainer (Cat. No. 352350, Corning) and washed with R10 media (RPMI 1640 supplemented with 10% fetal bovine serum) on ice. The remaining solid tissue was digested for 25 min at 37 °C and was filtered as described above. R10 media supplemented with EDTA (2 mmol/L) were then added to the collected cells. Single-cell suspensions were layered on a density gradient using Histopaque-1077 (Cat. No. 10771, Sigma-Aldrich) and then centrifuged at 2000*g* for 20 min at 4 °C. Following centrifugation, the upper 75% of the total volume was collected to exclude myocytes and cellular debris from the mononuclear fraction. Saline was then added to dilute the collected gradient solution, followed by centrifugation of the cell suspension at 2000*g* for 10 min at 4 °C. The pelleted cells were resuspended in 400 μL of cold Flow Cytometry Staining Buffer Solution (Cat. No. 00-4222-47, eBioscience) and processed for flow cytometry analysis as described below.

2.5. Isolation of splenocytes and peripheral blood cells

Spleens were removed from WT and DCM mice and placed in a 35-mm cell culture dish (Cat. No. 353001, BD Biosciences) with 5 mL DMEM culture medium (Gibco, Invitrogen). Splenocytes and peripheral blood cells were isolated from WT and DCM mice as previously described [7]. Briefly, spleens were perfused with 5 mL of DMEM at multiple locations to disperse single cells. The final cell suspension was centrifuged at 150*g* for 5 min at 4 °C, and the pellet was treated with 0.4 mL of RBC lysis buffer for 5 min at room temperature. Washed splenocytes were then resuspended in FACS staining buffer.

Peripheral blood cells were isolated from approximately 500 μL of peripheral blood collected in BD Microtainer Plastic Capillary Blood Collectors with Dipotassium EDTA (Cat. No. 02-669-38, Thermo Fisher Scientific). Erythrocytes were lysed with 1 mL of 1× RBC Lysis Buffer Solution (Cat. No. 00-4333-57, eBioscience) for 5 min at 37 °C. The lysis reaction was neutralized with 10 mL of saline. Peripheral blood leukocytes were collected by centrifugation at 500*g* for 5 min. The resulting pellets were resuspended in 400 μL of cold Flow Cytometry Staining Buffer Solution and processed for flow cytometry analysis as described below.

2.6. Flow cytometry

Isolated cell suspensions from WT and DCM hearts, spleens and peripheral blood (described above) were incubated for 30 min on ice with TruStain fcX (anti-mouse CD16/32) Antibody (Cat. No. 101319, BioLegend) to block Fc receptors. Samples were then incubated with a cocktail of fluorophore-labeled monoclonal antibodies (described below) for 1 h at 4 °C protected from light. Following a wash with cold saline, samples were centrifuged at 2000 rpm for 2 min at 4 °C