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Mast cell-deficiency protects mice from streptozotocin-induced diabetic cardiomyopathy

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Abstract

Mast cells (MCs) have been implicated in the pathogenesis of cardiometabolic diseases by releasing pro-inflammatory mediators. Patients and animals with diabetic cardiomyopathy (DCM) also show inflammatory cell accumulation in the heart. Here we detected MCs in mouse heart after streptozotocin (STZ)-induced DCM. DCM production caused significant systole and diastole interventricular septum and left ventricular (LV) posterior wall thinning, and systolic LV internal dilation in wild-type (WT) mice. DCM production also led to significant reductions of fractional shortening percentage, heart rate, body weight, heart weight, and significant increases of kidney, pancreas, and lung weight to body weight ratios, and blood hemoglobin HbA1c and glucose levels in WT mice. All these changes were improved or disappeared in MC-deficient *Kit^{W-sh/W-sh}* mice. In the myocardium from WT DCM mice, we detected significant decrease of cardiac cell proliferation and increases of cardiac cell death, chemokine expression, macrophage infiltration, inflammatory cytokine expression, and collagen deposition. These changes were also improved or disappeared in *Kit^{W-sh/W-sh}* DCM mice. Adoptive transfer of bone-marrow-derived MCs (BMMCs) from WT mice fully or partially reversed these cardiac functional and morphologic changes in *Kit^{W-sh/W-sh}* DCM recipient mice. Yet, adoptive transfer of BMMCs from *Il6^{-/-}* and *Tnf^{-/-}* mice failed to make these corrections or at much less extent than the WT BMMCs. Mechanistic studies demonstrated a role of MC and MC-derived IL6 and TNF- α in promoting cardiomyocyte death and cardiac fibroblast TGF- β signaling, and collagen synthesis and

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deposition. Therefore, MC inhibition may have therapeutic potential in attenuating DCM progression.

Keywords

Mast cell; Streptozotocin; Diabetic cardiomyopathy; Cardiomyocyte; Cardiac fibroblast

INTRODUCTION

Diabetic cardiomyopathy (DCM) is a clinical condition diagnosed when ventricular dysfunction develops in patients with diabetes in the absence of coronary atherosclerosis and hypertension.¹ Diabetic patients often develop early structural and functional cardiac changes such as left ventricle (LV) wall thickening, LV mass index increase, ejection fraction (EF) reduction, and increase in diastolic diameter.² In addition to these clinical manifestations, pathophysiological changes of DCM include cardiac oxidative stress, intramyocardial inflammation, microangiopathy, endothelial dysfunction, cardiac fibrosis, and increases of cardiac cytokine levels,^{3–5} along with increased myocardial cell apoptosis and decreased cardiac proliferation, all which can be triggered by the diabetic milieu^{3,5,6} or hyperglycemia.⁷ Mice receiving streptozotocin (STZ) treatment exert similar clinical and pathological changes to those in DCM patients. In mice, STZ induces systolic and diastolic dysfunction,^{8–10} accumulation of connective tissue collagen^{11,12} and cardiac interstitial fibrosis,¹³ along with body weight decrease and serum glucose increase.⁶ Inflammatory cell infiltration is one of the most prominent pathologic events in DCM. These infiltrates, such as macrophages, are essential sources of inflammatory cytokines that stimulate the expression of myocardial adhesion molecules ICAM-1 (intercellular adhesion molecule-I) and VCAM-1 (vascular cell adhesion molecule-1),¹⁴ and promote myocardial fibroblast collagen expression.⁴ Such immunocompetent infiltrates and associated inflammatory cytokines have been confirmed in DCM animals and patients.¹⁵

Like macrophages, mast cells (MCs) are inflammatory cells that also migrate to the site of inflammation. These cells have recently been found in human and mouse atherosclerotic lesions and white adipose tissues after disease initiation.^{16,17} Presence of MCs in the aortas or adipose tissues may produce chemokines as a feedback mechanism for consequent recruitment of macrophages and other inflammatory cells.¹⁸ Yet, there is no direct evidence whether MCs are important composite of these inflammatory infiltrates in the myocardium from DCM patients or animals. Using MC-deficient *Kit^{W-sh/W-sh}* mice, we demonstrated that the absence of MCs protected mice from diet-induced atherosclerosis, obesity, and type-2 diabetes.^{16,17} MCs participate in these vascular diseases and metabolic disorders by secreting inflammatory cytokines interferon- γ (IFN- γ) and interleukin-6 (IL6).

The pathologies of DCM share many similarities with atherosclerosis and obesity, in which endothelial cell adhesion molecule expression, inflammatory cell infiltration, cytokine production, matrix protein production, and cell apoptosis/proliferation play deleterious roles in the pathogenesis. In this study, we used MC-deficient mice and MC adoptive transfer

technique to establish a direct participation of these inflammatory cells in STZ-induced mouse DCM.

MATERIALS AND METHODS

Mice and DCM development

Mouse DCM was produced as previously described.^{8–10} Briefly, 8–10 weeks old wild-type (WT) and MC-deficiency *Kit^{W-sh/W-sh}* male mice all in C57BL/6 background between 23–25 g received intraperitoneal (i.p.) injections of 50 mg/kg streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO) dissolved in 100 mM pH 4.5 citrate buffer, for five consecutive days. Mice treated with buffer alone were used as controls. Mice were characterized at 28 days after the STZ treatment. ELISA determined blood glycated hemoglobin HbA1c levels (Cat# OKEH00661, Aviva Systems Biology Corporation, San Diego, CA). Blood glucose levels were measured with an Ascensia Contour Glucometer (Bayer HealthCare, Hippianny, NJ). There were a total of seven study groups: 1) WT mice untreated control, 2) WT with STZ treatment, 3) *Kit^{W-sh/W-sh}* mice untreated control, 4) *Kit^{W-sh/W-sh}* mice treated with STZ, 5) *Kit^{W-sh/W-sh}* mice repopulated with bone-marrow-derived MCs (BMMCs) from WT mice and then treated with STZ, 6) *Kit^{W-sh/W-sh}* mice received BMMCs from *Il6^{-/-}* mice and then with STZ treatment, and 7) *Kit^{W-sh/W-sh}* mice received BMMCs from *Tnf^{-/-}* mice and then with STZ treatment. BMC reconstitution was performed by intravenous tail vein injection of 1×10^7 BMCs per recipient mouse one day before STZ injection. Each group contained 7–14 mice. All mouse experiments were preapproved by the Institutional Animal Care and Use Committee, Brigham and Women's Hospital, protocol # 2016N000442.

Echocardiography

Transthoracic echocardiography was performed in conscious mice at 28 days after STZ treatment with the VisualSonics' Vevo 770 system (Ontario, Canada) that had the scanhead with a fixed focus without color Doppler. The heart was imaged in the 2-dimensional parasternal short-axis view, and an M-mode echocardiogram of the mid-ventricle was recorded at the level of the papillary muscles. Heart rate, posterior and interventricular septum wall thicknesses, and LV end-diastolic and end-systolic internal dimensions were measured from the M-mode image. LV fractional shortening (FS), used as an index of cardiac contractile function was defined as the end-diastolic diameter minus the end-systolic diameter normalized for the end-diastolic diameter.

Morphometric analysis

After the physiological analyses, mice were euthanized and their hearts were excised. Heart, lung, kidney, and pancreas weights were recorded immediately after isolation. LV was cut from apex to base into 3 transverse sections, and the medium parts were embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA) for 6 μ m frozen section preparation.

BMMC culture

WT (Cat# 000664, C57BL/6), *Il6^{-/-}* (Cat#002650, C57BL/6, N11) and *Tnf^{-/-}* (Cat#005540, B6/129S6, N1) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Congenic *Tnf^{-/-}* (C56BL/6, N10) mice were generated by back crossing to the C57BL/6

background. To prepare BMMCs, bone marrow cells from these mice were differentiated for 5 weeks in the presence of mouse IL3 (Cat#500-P53, PeproTech, Rocky Hill, NJ) and stem cell factor (Cat# AF-250-03, PeproTech) as described.^{16,17} MC purity reached to >95%, which was confirmed with toluidine blue staining for the presence of metachromatic granules, and flow cytometric analysis for cell surface expression of CD117 (1:200, Cat#553356, BD Biosciences, San Jose, CA) and FcεR-1α (1:200, Cat#MCA6066F, Bio-Rad, Hercules, CA), but absence of Sca-1 (1:200, Cat#553108, BD Biosciences) expression.

Real-time polymerase chain reaction (RT-PCR)

RT-PCR was used to determine ICAM-1, VCAM-1, TNF-α (tumor necrosis factor-α), IL6, lysyl oxidase mRNA levels in heart tissues. Total RNA was extracted from heart tissue using TRIzol reagent (Cat#15596018, Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Cat#EN0521, Ambion, Austin, TX) to remove genomic DNA contaminants. Equal amounts of RNA were reverse-transcribed, and quantitative PCR was assessed in a single-color RT-PCR detection system (Stratagene, La Jolla, CA). The mRNA level of each gene was normalized to β-actin.

Cell lysate immunoblot analysis

Cells were lysed in a RIPA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, proteinase inhibitor (Cat#11836170001, Roche Diagnostics Corporation, Indianapolis, IN), and phosphatase inhibitor cocktail (Cat#78442, Fisher Scientific, Hampton, NH). Cell lysates were centrifuged at 20,000 g for 15 min. Supernatant was collected for protein concentration determination using the BCA protein assay kit (Cat#PI23223, Thermo Fisher Scientific, Waltham, MA). The extracts were separated on SDS-PAGE gels by electrophoresis and transferred onto PVDF membranes (Cat#IPVH00010, Millipore, Billerica, MA), blotted and detected with different antibodies, including type I and III collagens, p-Smad2/3, Smad2/3, α-smooth muscle actin (α-SMA), Bax, Bid-tBid, cleaved caspase-3, and β-actin as detailed below.

Immunohistology

Heart frozen sections (6 μm) were prepared and immunostained for Ki67 (cell proliferation, 1:750, Cat#vp-RM04, Vector Laboratories, Burlingame, CA), Mac-3 (macrophage, 1:900, Cat#553322, BD Biosciences), CD4 (T-cell, 1:100, Cat#553043, BD Biosciences), and CD8a (T-cell, 1:100, Cat#14008185, Thermo Fisher Scientific). Sirius red staining was also performed to detect both type-I and III collagens as we previously described.^{19,20} Briefly, formalin-fixed frozen sections were incubated for 4 hours in a freshly prepared 0.1% solution of Sirius Red F3BA (Polysciences Inc., Warrington, Pennsylvania, USA) in saturated aqueous picric acid. After rinsing twice in 0.01 N HCl and distilled water, sections were briefly dehydrated and mounted in Permount (Vector Laboratories). Sirius red staining was analyzed by polarization microscopy. Apoptotic cells were detected using ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit according to the manufacturer (Cat#S7100, Millipore). Four or five fields from each section were randomly selected for analysis blindly. Cell-type specific apoptosis was also detected by immunofluorescent double staining of mouse heart sections with antibodies against cleaved caspase-3 (1:100, Cat#8172, Cell Signaling Technology, Danvers, MA) together with antibodies against myosin heavy chain

(MHC, cardiomyocyte, 1:100, Cat#bs-15444R-A488, Bioss Inc, Woburn, MA) and FITC-conjugated α -SMA (cardiac fibroblast, 1:500, Cat#F3777, Sigma-Aldrich).

Neonatal cardiomyocyte culture and apoptosis assay

Mouse neonatal cardiomyocyte primary culture was prepared as described.²¹ Briefly, hearts from neonatal mice were minced and dissociated with 0.10 % trypsin (Cat#15090046, Thermo Fisher Scientific) in DMEM followed by incubation on a 25-cm² flask for 30 min. Cells were then seeded onto a 6-well plate (2×10^5 cells/ml) in DMEM supplemented with 5% FBS (Cat#16000044, Thermo Fisher Scientific) and 0.1 mmol/L of 5-bromo-2-deoxyuridine (Cat#B-5002, Sigma-Aldrich) for 24 hours to prevent any residual nonmyocardial cell proliferation. Cells were then cultured in DMEM containing 0.1% bovine serum albumin (BSA, Cat#A-4919), 2 μ g/ml vitamin B12 (Cat#V-6629), 10 μ g/ml insulin (Cat#I-1882) all from Sigma-Aldrich, 10 μ g/ml transferrin (Cat#11108016, Thermo Fisher Scientific), and 5% FBS with or without 3×10^6 BMMCs from WT, *Il6*^{-/-}, and *Tnf*^{-/-} mice for 24 hours. Cardiomyocytes were then washed with PBS, fixed with 4% paraformaldehyde, and immunostained with the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Cat#S7100, Millipore).

Adult mouse cardiomyocyte and cardiac fibroblast culture

Adult mouse cardiomyocytes were prepared as described.²² Briefly, the mouse heart was excised and then perfused with a calcium-free and high potassium buffer followed by 0.1% type II collagenase digestion (Cat#LS004177, Worthington Biochem, Lakewood, NJ). Cardiomyocytes were incubated in MEM medium containing 1% penicillin/streptomycin (10,000 U/ml), 5 mM 2,3-butanedione monoxime, and 1 mg/ml BSA in a 2% CO₂ incubator at 37 °C. One day after equilibration, cells were treated with or without different amount of BMMCs ($1 \times 10^5 \sim 5 \times 10^5$ cells/well) for 4 hours. Hydrogen peroxide (H₂O₂, 135 μ M) was used as a positive control. Cells were photographed and collected for immunoblot analysis to detect the production of Bax (1:1000, Cat#2772s, Cell Signaling Technology), Bid (1:500, Cat#MAB860, R&D Systems, Minneapolis, MN), cleaved caspase-3 (1:1000, Cat#9661s, Cell Signaling Technology), and β -actin (1:3000, Cat#8457s, Cell Signaling Technology).

Adult mouse cardiac fibroblasts were prepared as described.²² After centrifugation from the cardiomyocyte isolation, cardiomyocytes precipitated in the bottom. The supernatant was centrifuged at 1,500 rpm for 5 min to obtain cardiac fibroblasts. Cells were cultured in a 5% CO₂ incubator at 37 °C for up to 3 days. Cells were then treated with or without WT BMMCs (1×10^5 cells/well) for 0.5, 1, 12, and 24 hours, followed by immunoblot analysis to detect p-Smad2 (1:1000, Cat#ab3849-I, Millipore), Smad2 (1:1000, Cat#5339s, Cell Signaling Technology), p-Smad3 (1:2000, Cat#ab52903, Abcam, Cambridge, MA), Smad3 (1:1000, Cat#9523s, Cell Signaling Technology), α -SMA (1:1000, Cat#14968s, Cell Signaling Technology), collagen-I (1:1000, Cat#PA5-29569, Thermo Fisher Scientific), collagen-III (1:1000, Cat#PA5-27828, Thermo Fisher Scientific), and β -actin (1:3000, Cat#8457s, Cell Signaling Technology).

Statistical analysis

All data were expressed as mean±SEM. For mouse heart protein sample ELISA and RT-PCR data, those with normal distribution and homogeneity of variance, independent *t* test, and one-way analysis of variance (ANOVA) LSD test were used for paired and multi-group comparisons. Non-parametric Mann-Whitney *U* test followed by Bonferroni correction was used for all other mouse data analysis due to small sample size and abnormal data distribution. SPSS16 version was used for analysis and *P*<0.05 were considered significant.

RESULTS

MC-deficiency protects mice from STZ-induced DCM

Prior studies showed that MCs in injured cardiac tissues advance cardiac adverse remodeling.²³ To generate mouse model of DCM, we first induced type-1 diabetes in mice by giving STZ i.p. injection to mice for five consecutive days as reported.^{8–10} At 28 days after STZ treatment, we harvested mouse heart. Frozen heart section staining with toluidine blue detected increased numbers of MCs in the myocardium, whereas the same preparation from untreated mice showed negligible MCs (Fig 1A/1B). These observations suggest an involvement of MCs in STZ-induced mouse DCM. To test this hypothesis, we treated both MC-sufficient WT mice and MC-deficient *Kit^{W-sh/W-sh}* mice with or without STZ. At 28 days after STZ treatment, echocardiogram showed that STZ-treated WT mice displayed significantly reduced diastolic and systolic interventricular septum (IVS) thickness and LV posterior wall (LVPW) thickness, increased systolic LV internal diameter (LVID), reduced LV fractional shortening percentage (FS%), and heart rate (HR), compared to those of WT control mice (Table 1). These parameters did not differ between WT and *Kit^{W-sh/W-sh}* vehicle-treated control mice. *Kit^{W-sh/W-sh}* mice received STZ treatment only showed reduced diastolic and systolic IVS, but still significantly higher than those from STZ-treated WT mice. All other tested parameters did not change significantly in *Kit^{W-sh/W-sh}* mice with and without STZ treatment (Table 1). Due to the nature of the mechanical scanhead on the Vevo 770 model echocardiograph system (VisualSonics Inc), it had a fixed focus without the color Doppler. This fixed focus of scanhead made the positioning difficult for apical imaging and the acquiring of wave forms to obtain diastolic function data unreliable. Therefore, we were unable to collect data to reflect the diastolic function changes. Yet, reduced FS% in WT DCM mice at 28 days after STZ treatment suggests that the myopathic state of the diabetic hearts in these mice might have developed from diastolic dysfunction to late systolic dysfunction stage.²⁴ In contrast, STZ and vehicle treatments of *Kit^{W-sh/W-sh}* mice did not show differences in FS% at the 28-day time point (Table 1).

Prior studies established a role of MC IL6 and INF- γ , but not TNF- α in diet-induced atherosclerosis, obesity and diabetes.^{16,17} To confirm the action of MCs in DCM and to test a specific role of MC-derived cytokines in DCM pathogenesis, we performed adoptive transfer by repopulating BMMCs from WT, *Il6^{-/-}*, and *Tnf^{-/-}* mice to MC-deficient *Kit^{W-sh/W-sh}* recipient mice, followed by DCM production. Toluidine blue staining detected similar numbers of MCs in the myocardium from *Kit^{W-sh/W-sh}* recipient mice after mice received BMMCs from different donors (WT, *Il6^{-/-}*, and *Tnf^{-/-}* mice) followed by DCM production, although the MC numbers in these recipient mouse hearts were still many fewer

than those from WT DCM mice (Fig 1B). WT donor BMMCs increased *Kit*^{W-sh/W-sh} recipient mouse diastolic LVPW and systolic LVID, and decreased FS% to the same levels as those in STZ-treated WT mice. Donor BMMCs from *Il6*^{-/-} mice showed similar activities to those of WT BMMCs in *Kit*^{W-sh/W-sh} recipient mice. Yet, *Kit*^{W-sh/W-sh} mice received donor BMMCs from *Tnf*^{-/-} mice showed reduced diastolic and systolic LVID, increased systolic LVPW, and increased FS%, compared with those from mice received WT BMMCs (Table 1).

As expected, WT mice showed reduced body weight (BW), heart weight (HW), HW to BW ratio, and increased kidney weight (KW) and lung weight (LW) to BW ratios after DCM production, although the increase of pancreas weight (PW) did not affect the ratio to BW. In *Kit*^{W-sh/W-sh} mice, production of DCM also reduced HW, but still much heavier than that in WT-DCM mice. In these mice, DCM production did not change BW, and HW-BW, KW-BW, PW-BW, and LW-BW ratios (Table 2). When *Kit*^{W-sh/W-sh} mice received adoptive transfer of BMMCs from WT, *Il6*^{-/-}, and *Tnf*^{-/-} mice, most of these morphometric variables did not change significantly. Only HW remained lower in all three types of recipient mice than the control *Kit*^{W-sh/W-sh} mice. PW-BW and LW-BW ratios were reduced in *Kit*^{W-sh/W-sh} recipient mice after WT BMMC adoptive transfer (Table 2). Together, these results from echocardiogram and tissue weight study suggest an essential role of MCs and MC-derived IL6, TNF- α , and possibly other untested MC granule mediators in mouse DCM development.

MC-deficiency improves STZ-induced weight loss and hyperglycemia in diabetic mice

Hyperglycemia has long been implicated in cardiac remodeling.^{25,26} STZ induces type-I diabetes.⁸⁻¹⁰ During the 28-day period after STZ induction, we found that WT mice showed continuous and significant body weight loss. Yet, *Kit*^{W-sh/W-sh} DCM mice showed similar wave of body weight gain as those of WT and *Kit*^{W-sh/W-sh} control mice, although *Kit*^{W-sh/W-sh} DCM mice still showed significant body weight loss compared with the control *Kit*^{W-sh/W-sh} mice (Fig 1C). In *Kit*^{W-sh/W-sh} DCM mice, reconstitution of BMMCs from WT, *Il6*^{-/-}, and *Tnf*^{-/-} mice changed the body weight loss curve and showed significantly more body weight loss than the *Kit*^{W-sh/W-sh} DCM mice at the 28-day time point, but these losses did not reach to the levels of WT DCM mice. Body weight loss among the three groups of recipient mice did not differ (Fig 1C). At the 28-day time point after STZ treatment, all STZ-treated mice showed high plasma HbA1c and glucose levels, as previously reported.^{27,28} Yet, the increase of HbA1c levels in *Kit*^{W-sh/W-sh} DCM mice was blunted, but reversed in these mice after receiving *in vitro* prepared BMMCs from WT, *Il6*^{-/-}, and *Tnf*^{-/-} mice (Fig 1D). At this time point, reduced plasma glucose levels in *Kit*^{W-sh/W-sh} DCM mice were also increased again after mice received BMMCs from WT mice, but not those from *Il6*^{-/-} or *Tnf*^{-/-} mice (Fig 1E).

MC activity in cardiac cell apoptosis and proliferation

Cardiac cell apoptosis represents an important pathogenic mechanism of progressive DCM in humans and animals.²⁹ Protected cardiac morphologic and functional changes in MC-deficient DCM mice suggest reduced cardiac cell death in the myocardium from these mice. TUNEL staining tested this hypothesis and found greatly increased TUNEL-positive cells in

WT DCM mice, but blunted cell death in *Kit^{W-sh/W-sh}* mice with or without DCM. Adoptive transfer of BMMCs from WT mice, but not those from *Il6^{-/-}* or *Tnf^{-/-}* mice significantly increased the numbers of TUNEL-positive cells in the myocardium from *Kit^{W-sh/W-sh}* recipient mice (Fig 2A). Therefore, both IL6 and TNF- α from MCs contributed to cardiac cell apoptosis in DCM heart. To examine the apoptotic cell types in DCM mouse heart, we performed immunofluorescent double staining with antibodies against cleaved caspase-3 (cell apoptosis marker) together with cell-type markers MHC (cardiomyocyte) or α -SMA (cardiac fibroblast). We detected MHC- and cleaved caspase-3-double positive apoptotic cardiomyocytes in DCM mouse heart (Fig 2B, left panel), but none of these α -SMA-positive cardiac fibroblasts was positive for cleaved caspase-3 (Fig 2B, right panel).

Myocardial cell proliferation also occurs during DCM repair and cardiac regeneration.³⁰ Improved cardiac function in MC-deficient DCM mice suggests a role of MC in inhibiting ventricular cell proliferation. Immunostaining of heart sections with mouse Ki67 antibody demonstrated significant reduction of cardiac cell proliferation in heart from WT DCM mice, but such reduction was significantly less in *Kit^{W-sh/W-sh}* DCM mice (Fig 2C). Adoptive transfer of BMMCs from WT and *Il6^{-/-}* mice, but not those from *Tnf^{-/-}* mice significantly reduced cardiac cell proliferation (Fig 2C), suggesting an essential role of MCs and MC-derived TNF- α in cardiac cell proliferation.

MC activity in cardiomyocyte apoptosis

To test a direct role of MCs in cardiomyocyte apoptosis, we prepared both neonatal and adult mouse cardiomyocytes. Same amount of BMMCs from WT and *Il6^{-/-}* mice induced neonatal cardiomyocyte death. Yet, BMMCs from *Tnf^{-/-}* mice displayed much weaker activity in inducing neonatal cardiomyocyte death than those from WT and *Il6^{-/-}* mice (Fig 3A).

Hydrogen peroxide (H_2O_2) induced adult cardiomyocyte apoptosis and necrosis.^{31,32} WT BMMCs also induced adult cardiomyocyte death in the absence of H_2O_2 , although at a much weaker level than H_2O_2 . Different amount of WT BMMCs, 1×10^5 and 5×10^5 , did not show significant difference in inducing adult cardiomyocyte death by morphology quantification (Fig 3B). Western blot analysis showed that both doses of WT BMMCs increased the expression of Bax and Bid, production of tBid, and cleavage activation of caspase-3. It appears that high dose (5×10^5) of WT BMMCs produced more 19-kDa activated caspase-3 than the 17-kDa activated caspase-3 than low dose (1×10^5) of WT BMMCs did (Fig 3C). BMMC-induced adult cardiomyocyte death was significantly blunted when BMMCs from *Il6^{-/-}* and *Tnf^{-/-}* mice were used (Fig 3D), suggesting that both IL6 and TNF- α from MCs are essential to induce adult cardiomyocyte death, consistent to the observation from the myocardium TUNEL staining (Fig 2A). Western blot analysis showed similar results. WT BMMCs induced the expression of Bax and Bid, and increased the production of tBid and cleaved caspase-3. Bid expression and tBid production were reduced to the baseline (untreated cardiomyocytes) in cells treated with BMMCs from *Tnf^{-/-}* mice. The production of cleaved caspase-3 in cardiomyocytes treated with BMMCs from *Il6^{-/-}* or *Tnf^{-/-}* mice did not differ from that from untreated cardiomyocytes (Fig 3E).

MC function in myocardial inflammatory cell infiltration

Inflammatory cells appear at site of inflammation, such as adipose tissue,^{33,34} vascular lesions,¹⁷ and injured myocardium.³⁵ This pathologic change is affected by altered expression of chemokines and adhesion molecules,⁴ which promote inflammatory cell adhesion and migration. In myocardium from WT DCM mice, we detected significantly higher expression level of adhesion molecule ICAM-1 than that from the WT control mice. MC-deficiency blunted the increase of ICAM-1 expression. In MC-deficient *Kit^{W-sh/W-sh}* DCM mice, adoptive transfer of BMMCs from WT and *Il6^{-/-}* mice, but not those from *Tnf^{-/-}* mice reversed the increase of myocardial ICAM-1 expression (Fig 4A). The expression of another adhesion molecule VCAM-1 did not show significant differences between the groups (Fig 4B).

Different expression levels of adhesion molecules in the myocardium may affect inflammatory cell infiltration differently to the heart. STZ treatment induces cardiac macrophage infiltration.³⁶ Mac-3-positive macrophage accumulation was significantly increased in WT DCM mouse hearts. Yet, DCM production in *Kit^{W-sh/W-sh}* mice did not show significant increase of heart macrophage contents (Fig 4C). Reconstitution of WT BMMCs but not BMMCs from *Il6^{-/-}* and *Tnf^{-/-}* mice reversed heart macrophage accumulation. Different from macrophages, DCM development tended to reduce myocardial CD4⁺ T cells and had no effect on CD8⁺ T-cell accumulation, similar to what were reported previously.^{37,38} Yet, both CD4⁺ and CD8⁺ T cells were significantly higher in hearts from *Kit^{W-sh/W-sh}* control mice than those in WT control mice. DCM production in *Kit^{W-sh/W-sh}* mice reduced heart CD4⁺ T cells, but had no effect on CD8⁺ T cells. Reconstitution of BMMCs from WT and *Tnf^{-/-}* mice, but not those from *Il6^{-/-}* mice reduced DCM heart CD4⁺ and CD8⁺ T cells (Fig 4D/4E). Therefore, MCs may exert different activities on macrophages versus lymphocytes.

MC activity in inflammatory cytokine expression in DCM heart

As a consequence, accumulation of MCs (Fig 1A/1B) and macrophages (Fig 4C) in DCM hearts may release inflammatory cytokines to modulate cardiac cell death (Fig 2A/2B and 3), proliferation (Fig 2C) and fibrosis. RT-PCR analysis of heart tissue revealed significant increases of both TNF- α and IL6 in WT DCM mice compared with WT control mice. Such increases were blunted in *Kit^{W-sh/W-sh}* mice with or without DCM (Fig 5A/5B). In *Kit^{W-sh/W-sh}* DCM mice, reconstitution of WT BMMCs effectively increased myocardial expression of both TNF- α and IL6. In contrast, donor BMMCs from *Il6^{-/-}* and *Tnf^{-/-}* mice failed to change myocardial IL6 expression in *Kit^{W-sh/W-sh}* recipient DCM mice, and only BMMCs from *Il6^{-/-}* mice but not those from *Tnf^{-/-}* mice increased recipient mouse heart TNF- α expression (Fig 5A/5B).

Heart tissue extract ELSA analysis yielded same conclusions. Production of DCM in WT mice, but not in *Kit^{W-sh/W-sh}* mice increased heart TNF- α and IL6 protein levels. In *Kit^{W-sh/W-sh}* recipient DCM mice, donor BMMCs from WT mice, but not those from *Il6^{-/-}* or *Tnf^{-/-}* mice reversed heart TNF- α and IL6 levels (Fig 5C/5D). These observations are consistent to prior studies that MCs release TNF- α , IL6, and many other untested mediators to trigger myocardial inflammation.³⁹

MC-deficiency reduces DCM mouse LV collagen deposition and fibrosis

Type-I and type-III collagens abound in the LV and accumulate during DCM development. Uncontrolled synthesis and deposition lead to ventricular adverse remodeling.^{11,12} Lysyl oxidase (Lox) activity is required for the collagen fibril architecture.⁴⁰ Impaired cardiac function in WT DCM mice and protected cardiac function in *Kit^{W-sh/W-sh}* DCM mice suggest a role of MC in collagen synthesis and fibrillation. Sirius red staining allowed detection of collagen-I and III under a polarized light microscope. In hearts from WT mice, DCM production increased the deposition of both collagen-I and III. Yet, such change was not evident in *Kit^{W-sh/W-sh}* DCM mice (Fig 6A). Reconstitution of BMMCs from WT, *Il6^{-/-}* and *Tnf^{-/-}* mice increased myocardial type-I and type-III collagens at different levels, but only *Il6^{-/-}* BMMC-reconstituted hearts reached statistic significance in collagen-I increase compared with *Kit^{W-sh/W-sh}* DCM control mice. WT and *Tnf^{-/-}* BMMC-reconstituted hearts also showed increased collagen-I, but did not reach the significance, $P=0.062$ and $P=0.060$, respectively (Fig 6A, left panel). Only BMMCs from WT mice, but not those from *Il6^{-/-}* and *Tnf^{-/-}* mice showed a trend of myocardial collagen-III increase. Statistic analysis did not show the significance (Fig 6A, middle panel). As expected, Lox expression at the mRNA level was increased in WT DCM mice. Such increase was again blunted in *Kit^{W-sh/W-sh}* DCM mice (Fig 6B). Reconstitution of BMMCs from WT and *Il6^{-/-}* mice, but not those from *Tnf^{-/-}* mice reversed the Lox expression in *Kit^{W-sh/W-sh}* DCM mice (Fig 6B). These observations suggest that MC-derived TNF- α and IL6 both contributed to myocardial collagen deposition and fibrosis.

To confirm further a role of MC in cardiac fibrosis, we treated cardiac fibroblasts with WT BMMCs in the absence of exogenous TGF- β . At the early time points, 30 min to 1 hour, we detected increased phosphorylation of TGF- β downstream signaling molecules Smad2 and Smad3. At the later time points, 12 to 24 hours after WT BMMC treatment, both p-Smad2 and p-Smad3 expression reduced, but the expression of all three fibrotic proteins α -SMA, and collagen-I and III increased (Fig 6C). These observations established a direct role of MC in activating cardiac fibroblasts and promoting the expression of collagen and α -SMA.

DISCUSSION

Accumulating evidences suggest a role of MCs in cardiac adverse remodeling.⁴¹ Earlier observations of retrospective and prospective studies showed that histamine H2-receptor antagonist famotidine reduced LV end-diastolic and end-systolic lengths and plasma B-type natriuretic peptide (BNP, as a marker of cardiac hypertrophy) level in patients with chronic heart failure. Although famotidine did not affect significantly the FS%, patients treated with this antagonist had greatly reduced readmission rate because of worsening of heart failure,⁴² suggesting an involvement of MCs in cardiac damage during heart failure development. MC accumulation has also been reported in DCM hearts from humans and animals.^{23,43} In BioBreeding rats and in STZ-treated diabetic mice, MC inhibition with cromolyn or nedocromil reduced the myocardial production of inflammatory cytokines and collagens and delayed the disease onset.^{43,44} Here we presented a direct evidence of MC activity in the pathogenesis of STZ-induced mouse DCM using the MC-deficient mice and MC

reconstitution technique, which allowed us to test a specific role of IL6 and TNF- α from MCs in DCM.

Mouse experimental DCM models include pancreatic toxin-, diet- and genetic mutation-induced type-1 diabetes (T1D) and type-2 diabetes (T2D). Pancreatic toxin-induced T1D occurs in few days, whereas diet-fed or genetic mutant mice may develop diabetes in weeks to months that may better reflect the human scenario.⁴⁵ Although human diabetes never occurs in days, STZ-induced DCM has been broadly used^{8–10,45–47} because STZ-administration could recapitulate the most human DCM features, including reductions in systolic and diastolic function and increase of cardiac hypertrophy and fibrosis. This study did not test MC function in long-term DCM models, such as non-obese diabetic (NOD) mice and Akita mice that develop T1D and hyperglycemia in 6~30 week, or *ob/ob*, *db/db*, and diet-induced obese (DIO) mice that develop T2D and hyperglycemia in weeks.⁴⁵ We previously showed that MC-deficiency reduced diabetes in DIO mice¹⁶ but increased diabetes in *ob/ob* mice because leptin-deficiency altered MC functions.⁴⁸ Therefore, MC functions in these long-term DCM models remain to be explored.

STZ induces diabetes by targeting β cells in the pancreas.^{47,49} MC accumulation in the islet also promotes β -cell apoptosis.⁵⁰ In isolated human islets and INS-1E cells, histamine induced β -cell apoptosis in a caspase-independent mechanism,⁵⁰ suggesting a role of MCs in islet β -cell apoptosis. Blunted body weight loss and reduced blood HbA1c and glucose levels in MC-deficient *Kit^{W-sh/W-sh}* DCM mice relative to WT DCM mice (Fig 1C-1E) indirectly supported this mechanism. Although there are few conflict reports against the hypothesis of MC involvement in T1D, some of them are contaminated with experimental designs. For example, anti-Fc ϵ R1 antibody treatment in non-obese diabetic (NOD) mice activated MCs but delayed the onset of T1D.⁵¹ Our recent studies showed that not only MCs but also macrophages, and even vascular cells also express Fc ϵ R, the high affinity receptor for immunoglobulin E (IgE).⁵² Anti-Fc ϵ R1 antibody treatment may have at least two mechanisms in regulating these Fc ϵ R-positive vascular and inflammatory cells: one is to bind the cell surface Fc ϵ R for cell activation and another is to block IgE function, thereby inactivating the Fc ϵ R-positive cells. A direct test of the role of MCs in T1D is probably to use MC-deficient model. One major potential concern of MC-deficient model is the changes in number and activity of other immune cells, which may be affected by MC-deficiency.⁵³ That is why we believe that MC repopulation in MC-deficient mice becomes essential to claim a role of MCs in DCM.

MC activities on cardiac adverse remodeling in DCM patients or animals can be complicated due to the complexity of the MC granules, such as histamine, cytokines, chemokines, growth factors, and proteases. In DCM hearts, MC mediators may directly act on target cells such as cardiomyocytes and cardiac fibroblasts or extracellular matrix (ECM). Our study demonstrated increase of myocardial collagen I and III protein levels and Lox mRNA level in WT DCM mice but not in *Kit^{W-sh/W-sh}* DCM mice (Fig 6A/6B). MC chymase and tryptase may contribute directly to these changes. With unknown mechanisms, tryptase induced collagen synthesis and proliferation of cardiac fibroblasts from spontaneously hypertensive rats.⁵⁴ Chymase inhibitor suppressed collagen expression in the

myocardium from dogs with heart failure⁵⁵ and chymase directly increased the expression of TGF- β 1 p-Smad2/3 in rat cardiac fibroblasts.⁵⁶

MCs may also contribute to increased cardiomyocyte apoptosis, reduced cardiac cell proliferation, increased myocardial collagen expression and deposition, and cardiac dysfunction in DCM mice *via* indirect mechanisms by releasing inflammatory mediators. Here we used BMMCs from *Il6*^{-/-} and *Tnf*^{-/-} mice along with those from WT mice to test an indirect role of these MC cytokines on cardiomyocyte death, cardiac fibroblast collagen production, cardiac cell proliferation, myocardial macrophage infiltration, and inflammatory cytokine expression. Our earlier studies from vascular diseases established a role of MC-derived interferon- γ and IL6, but not TNF- α in stimulating vascular cell protease expression and arterial wall remodeling.^{17,57} Different from those in the aortic wall, cardiac MCs are a major source of TNF- α .⁵⁸ In TNF- α -deficient mice, descending aortic banding-induced cardiac apoptosis, hypertrophy, inflammatory response, and reparative fibrosis were all attenuated.⁵⁹ Compared to BMMCs from *Il6*^{-/-} mice, those from *Tnf*^{-/-} mice showed less activities in inducing neonatal cardiomyocyte death (Fig 3A) and much weaker activity in promoting the production of Bax, Bid, tBid, and cleaved caspase-3 in adult cardiomyocytes (Fig 3E). As we summarized recently, cysteinyl cathepsins promote cell death by producing tBid from Bid.⁶⁰ Although not tested in this study, MCs may release TNF- α , IL6, and many other cytokines to induce cardiomyocyte expression of cysteinyl cathepsins for the production of pro-apoptotic tBid^{60,61} and activation of caspase-3.⁶² MC activity in cardiac fibrosis was first tested in *Kit*^{W/W^v} mice after mice were exposed to abdominal aortic banding with blunted perivascular fibrosis.⁵⁸ MC-derived TNF- α has been well studied in promoting cardiac fibrosis. Cardiac fibrosis and dysfunction developed in hearts from mice with cardiac-restricted TNF- α overexpression, but not in hearts from MC-deficient (*Kit*^{W-sh/W-sh}) TNF- α overexpression mice, indicating a role of MC-derived TNF- α in cardiac fibrosis.⁶³ Indeed, hearts from patients with DCM contain high levels of cathepsins S, K, B, L and their inhibitor cystatin C.⁶⁴ These proteases can come from MCs (Fig 1B), macrophages (Fig 4C), or other inflammatory infiltrates. Cardiomyocytes and fibroblasts also express cathepsins after exposure to MC granules. We demonstrated recently that MC chymase increased the expression of cathepsins S and K, and matrix metalloproteinase (MMP)-2 and -9 in cardiomyocytes, macrophages, and T cells (Shi, unpublished observations). Cathepsin expression in cardiomyocytes may contribute to their apoptosis in DCM hearts (Fig 2A/2B). Many of these cathepsins from MCs, other inflammatory cells, cardiomyocytes, and cardiac fibroblasts are strong collagenases that may mediate myocardial ECM degradation. Yet, the collagenase activity of cathepsins may not explain reduced collagen contents in DCM hearts from *Kit*^{W-sh/W-sh} mice (Fig 6A). Indeed, cathepsin L-deficiency leads to dilated cardiomyopathy.⁶⁵ We recently demonstrated that cathepsins in fibroblasts exert protease-independent activity in regulating TGF- β signaling and tissue fibrosis (Shi, unpublished observations). Different from cathepsins, mouse heart at six weeks after STZ-induced DCM shows reduced MMP-2 activity and MT1-MMP expression, but increased their inhibitor TIMP-2.⁶⁶ Both MMP-2 and MT1-MMP are collagenases.^{67,68} Reduced myocardial MMP-2 activity and MT1-MMP expression may contribute to elevated collagen deposition in these DCM hearts.⁶⁶

Together, this study established a direct role of MCs in STZ-induced mouse DCM and affirmed prior studies from MC inhibitor nedocromil, which effectively halted STZ-induced heart contractile dysfunction in diabetic mice.⁴³ Although a direct role of MCs in long-term models of experimental DCM has not been tested, MC inhibitor cromolyn delayed T1D onset in BioBreeding DRlyp/lyp rat, which develop diabetes in 12 weeks otherwise.⁴⁷ We previously showed that MC inhibitors cromolyn and ketotifen reduced diet-induced T2D in mice. All those MC inhibitors are over-the-counter drugs to treat patients with allergic diseases. Test of MC direct involvement in either short-term or long-term models of experimental DCM and successful improvement of diabetes and cardiac function after treatment with MC inhibitor suggest the potential of using these OTC anti-allergy drugs among DCM patients, a hypothesis that merits further pre-clinical and clinical studies.

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ABBREVIATIONS:

DCM	diabetic cardiomyopathy
MC	mast cell
STZ	streptozotocin
WT	wild-type
FS	fractional shortening
EF	ejection fraction
IVS	interventricular septum
LV	left ventricle
LVID	left ventricle internal diameter
LVPW	left ventricle posterior wall
α-SMA	α-smooth muscle actin
Lox	lysyl oxidase
ICAM-1	intercellular adhesion molecule-I
VCAM-1	vascular cell adhesion molecule-1
IFN-γ	interferon-γ
TNF-α	tumor necrosis factor-α

IL6	interleukin-6
BMMC	bone-marrow-derived mast cell
BSA	bovine serum albumin
HR	heart rate
BW	body weight
HW	heart weight
KW	kidney weight
PW	pancreas weight
LW	lung weights
HbA1c	hemoglobin A1c
MHC	myosin heavy chain
BNP	B-type natriuretic peptide
T1D	type-1 diabetes
T2D	type-2 diabetes
IgE	immunoglobulin E
DIO	diet-induced obesity
NOD	non-obese diabetic
ECM	extracellular matrix
MMP	matrix metalloproteinase
DAPI	4',6-diamidino-2-phenylindole

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At a Glance Commentary

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Background

Mast cells have been implicated in cardiovascular diseases by releasing inflammatory mediators after activation. Here we found mast cell accumulation in mouse heart with streptozotocin-induced diabetic cardiomyopathy. Using mast cell-deficient mice and bone marrow-derived mast cells from IL6- and TNF- α -deficient mice, we demonstrated that mast cells used IL6 and TNF- α and probably many other untested mediators to promote diabetic cardiomyopathy-like heart morphologic changes, cardiac dysfunctions, body weight loss, and blood hemoglobin A1c and glucose increase, and associated cardiomyocyte death, cardiac cell proliferation, myocardium adhesion molecule expression, macrophage accumulation and pro-inflammatory cytokine expression, and cardiac fibroblast TGF- β signaling and fibrosis.

Translational Significance

Interruption of mast cell activation may have therapeutic potential for patients with diabetic cardiomyopathy.

Fig 1. STZ-induced mouse DCM. **A.** Toluidine blue staining detected MCs (arrows) on heart sections from mice with and without DCM. Scale: 100 μ m. **B.** Quantification of heart toluidine blue-positive MCs. **C.** Body weight changes. **D.** Plasma HbA1c levels. **E.** Blood glucose level changes in mice with and without DCM and different treatments at indicated time points after STZ injection. n = 7~14 per group.

Fig 2.

Myocardial cell death and proliferation from DCM mice at 28 days after STZ treatment. **A.** Heart section TUNEL staining quantification for apoptotic cells from different groups of mice as indicated. **B.** Representative immunofluorescent double staining of cleaved caspase-3 with myosin heavy chain (cardiomyocyte) or α -SMA (cardiac fibroblast). Sections were counterstained with DAPI. Scale: 100 μ m. **C.** Heart section Ki67 staining and quantification for proliferating cells from different groups of mice as indicated. Representative images are shown to the right. Scale: 100 μ m. *n* = 7~14 per group.

Fig 3.

MC activity in neonatal and adult cardiomyocyte apoptosis. **A.** Mouse neonatal cardiomyocyte death induced with and without different types of BMMC lysates. $n=8\sim9$ per group. **B.** Mouse adult cardiomyocyte death after treatment with different amount (1×10^5 or 5×10^5) of WT BMMC lysates. Cardiomyocytes treated with and without H₂O₂ were used as controls. $n=11\sim13$ per group. **C.** Immunoblot analysis detected Bax, Bid-tBid, and cleaved caspase-3 in adult cardiomyocytes from different treatments. **D.** Mouse adult cardiomyocyte death induced with and without different BMMC lysates. $n=8\sim9$ per group. **E.** Immunoblot detected Bax, Bid-tBid, and cleaved-caspase-3 productions in adult cardiomyocytes from different treatments as indicated. Immunoblots were representative experiments from at least three repeats. Representative images for panels **A**, **B**, and **D** are shown to the right. Scale: 100 μm .

Fig 4.

MC activities on myocardial cell adhesion molecule expression and inflammatory cell accumulation in DCM heart. **A-B.** RT-PCR analysis of ICAM-1 and VCAM-1 in hearts from different groups of mice as indicated. **C-E.** Immunostaining detected Mac-3-positive macrophages and CD4-positive and CD8-positive T cells in frozen sections from different groups of mice as indicated. n = 7~14 per group.

Fig 5.

RT-PCR and ELISA detection of TNF- α and IL6 expression in hearts from DCM mice after different treatments. **A-B**. RT-PCR analysis of TNF- α and IL6 expression in hearts from different groups of mice as indicated. **C-D**. ELISA quantification of TNF- α and IL6 in heart tissue lysates from the same DCM mice as in **A-B**. $n = 7\sim 14$ per group.

Fig 6.

MC activities in TGF- β signaling and cardiac fibrosis. **A.** Sirius red staining detected heart type-I and type-III collagens from DCM mice with different treatments as indicated. Representative images (red: collagen I; green: collagen III) are shown to the right. Scale: 1 mm, inset scale: 400 μ m. **B.** RT-PCR analysis of mouse heart lysyl oxidase expression after different treatments as indicated. $n = 7\sim 14$ per group. **C.** Immunoblot analysis detected p-Smad2/3, Smad2/3, α -SMA, collagen-I and collagen-III expressions in adult mouse cardiac fibroblasts after treatment with WT BMMC lysates for different time points as indicated. Data are representative of at least three repeats.

Table 1.Cardiac function in WT and *Kit^{W-sh/W-sh}* mice at 28 days after STZ injection.

Variables	WT		<i>Kit^{W-sh/W-sh}</i>		<i>Kit^{W-sh/W-sh}</i> +BMMC		
	Control (n=12)	DCM (n=13)	Control (n=14)	DCM (n=14)	WT (n=13)	<i>Il6</i> ^{-/-} (n=7)	<i>Tnf</i> ^{-/-} (n=9)
IVS;d (mm)	0.80 ± 0.03	0.64 ± 0.03 *	0.84 ± 0.01	0.75 ± 0.02 *	0.76 ± 0.02 *	0.80 ± 0.04	0.80 ± 0.04 [#]
LVID;d (mm)	2.97 ± 0.05	2.96 ± 0.09	3.41 ± 0.04	3.27 ± 0.05	3.34 ± 0.08	3.33 ± 0.12	2.99 ± 0.09 ^{*#} ▲
LVPW;d (mm)	0.81 ± 0.03	0.70 ± 0.03 *	0.84 ± 0.02	0.78 ± 0.03	0.87 ± 0.03 [#]	0.84 ± 0.04 [#]	0.89 ± 0.04 [#]
IVS;s (mm)	1.23 ± 0.03	0.97 ± 0.07 *	1.38 ± 0.03	1.21 ± 0.05 *	1.19 ± 0.03 *	1.20 ± 0.02 *	1.31 ± 0.04
LVID;s (mm)	1.56 ± 0.05	1.90 ± 0.08 *	1.84 ± 0.06	1.83 ± 0.06	2.06 ± 0.11 ^{*#}	2.06 ± 0.10 ^{*#}	1.54 ± 0.06 ^{*#} ▲
LVPW;s (mm)	1.31 ± 0.04	1.01 ± 0.06	1.37 ± 0.03	1.25 ± 0.05	1.21 ± 0.05 *	1.22 ± 0.05 [#]	1.35 ± 0.04 ▲
FS%	47.54 ± 1.40	36.02 ± 1.91 *	45.98 ± 1.23	44.05 ± 1.54	38.86 ± 2.04 ^{*#}	39.79 ± 1.26 ^{*#}	48.49 ± 1.63 ▲
HR (bpm)	692.67 ± 14.89	525.69 ± 49.02 *	688.11 ± 6.58	631.64 ± 35.66	674.38 ± 8.23	699.07 ± 10.78	689.72 ± 10.27

* $P < 0.05$ vs corresponding controls $P < 0.05$ vs. WT-DCM[#] $P < 0.05$ vs. *Kit^{W-sh/W-sh}*-DCM▲ $P < 0.05$ vs. WT BMMC reconstituted mice.

One-way analysis of variance (ANOVA) followed by least significant difference (LSD) test.

Abbreviations: IVS;d: interventricular septum (diastole); LVID;d: left ventricular internal diameter (diastole); LVPW;d: left ventricular posterior wall (diastole); IVS;s: interventricular septum (systole); LVID;s: left ventricular internal diameter (systole); LVPW;s: left ventricular posterior wall (systole); FS: fractional shortening; HR: heart rate (beat per minute).

Table 2.

Morphometric analyses.

Variables	WT		<i>Kit^{W-sh/W-sh}</i>		<i>Kit^{W-sh/W-sh}</i> +BMMC		
	Control (n=12)	DCM (n=13)	Control (n=14)	DCM (n=14)	WT (n=13)	<i>Il6</i> ^{-/-} (n=7)	<i>Tnf</i> ^{-/-} (n=9)
BW (g)	25.03 ± 0.17	20.60 ± 1.21 *	28.20 ± 0.62	26.66 ± 0.89	27.26 ± 0.67	25.44 ± 1.46	25.11 ± 0.34
HW (mg)	124.08 ± 3.49	88.85 ± 7.00 *	136.06 ± 3.95	116.93 ± 3.98 *	121.85 ± 3.15 *	119.61 ± 6.19 *	120.64 ± 3.53 *
HW/BW (mg/g)	4.95 ± 0.14	4.29 ± 0.21 *	4.83 ± 0.10	4.57 ± 0.09	4.47 ± 0.06	4.71 ± 0.08	4.50 ± 0.12
KW/BW (mg/g)	14.12 ± 0.29	15.88 ± 0.64 *	14.92 ± 0.33	14.17 ± 0.20	14.92 ± 0.23	14.88 ± 0.39	15.01 ± 0.49
PW/BW (mg/g)	5.12 ± 0.14	6.28 ± 0.32	6.16 ± 0.31	6.49 ± 0.32	5.25 ± 0.22 #	6.07 ± 0.31	6.32 ± 0.21
LW/BW (mg/g)	4.88 ± 0.20	6.38 ± 0.39 *	4.83 ± 0.12	5.27 ± 0.16	4.87 ± 0.17 #	5.59 ± 0.27	5.26 ± 0.18

* $P < 0.05$ vs. corresponding control $P < 0.05$ vs. WT-DCM# $P < 0.05$ vs. *Kit^{W-sh/W-sh}* DCM.

One-way analysis of variance (ANOVA) followed by Student Newman-Keuls (SNK) test.

Abbreviations: BW: body weight, HW: heart weight; KW: kidney weight, PW: pancreas weight, LW: lung weight, HW/BW: heart weight to body weight ratio, KW/BW: kidney weight to body weight ratio, PW/BW: pancreas weight to body weight ratio, LW/BW: lung weight to body weight ratio.