The Effects of Extracellular Vesicles Derived From Krüppel-like Factor 2 Overexpressed Endothelial Cells on the Regulation of Cardiac Inflammation in the Dilated Cardiomyopathy

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Research

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Abstract

Background

Dilated cardiomyopathy (DCM) is one of the common causes of heart failure. Myocardial injury triggers an inflammatory response and recruits immune cells into the heart. High expression of Krüppel-like factor 2 (KLF2) in endothelial cells (ECs) could exert the anti-inflammatory effect. However, the role of extracellular vesicles from KLF2-overexpressed ECs (KLF2-EVs) in DCM remains unclear.

Methods and Results

EVs were separated from the supernatant of KLF2-overexpressed ECs by gradient centrifugation. Mice were administered with a repetitive low-dose doxorubicin (DOX) and then received KLF2- EVs through intravenous injection. Treatment with KLF2- EVs prevented doxorubicin-induced left ventricular dysfunction and reduced recruitment of Ly6$^{\text{high}}$ Mo/Mø in myocardium. To identify the mechanisms involved in this beneficial effect we used flow cytometry to detect Ly6$^{\text{high}}$ monocytes in bone marrow and spleen tissue. KLF2-EVs increased the retention of Ly6C$^{\text{high}}$ monocytes in the bone marrow, but not the spleen tissue. KLF2-EVs also significantly downregulated C-C chemokine receptor 2 (CCR2) protein expression in cells from the bone marrow.

Conclusion

EVs derived from KLF2-overexpressed ECs reduced cardiac inflammation and ameliorated left ventricular dysfunction in DCM mice, which is achieved by targeting the CCR2 protein to inhibit Ly6C$^{\text{high}}$ monocytes mobilizing from bone marrow.

Introduction

Dilated cardiomyopathy (DCM) is a disease characterized by progressive worsening of contractile dysfunction and ventricular dilation[1-4]. So far, the 5-year survival rate of DCM is less than 50% without specific treatment[5]. Therefore, it is crucial to explore the therapeutic strategy of DCM. Recently, several studies indicated that inflammatory response and immune cells played an important role in the process of DCM[6-8]. But the exact pathogenesis of DCM is still unclear.

The endothelial cells (ECs) lining all blood vessels and involving many physiological processes, such as homing immune cells to specific sites in the body, play a critical regulator in tissue homeostasis[9-11]. In previous studies, under the condition of laminar flow, KLF2 highly expressed in ECs which showed an anti-inflammatory phenotype[11, 12]. While under turbulent flow, KLF2 lowly expressed in the ECs which show a pro-inflammatory phenotype. Prior work from our group had revealed that KLF2-overexpressed ECs effectively preserved the anti-inflammatory phenotype and contributed to regulate immunity by secretion of extracellular vesicles (EVs)[13].
EVs, small vesicles with a diameter about 40-150nm, secreted by different living cells, such as ECs, macrophages, mesenchyme stem cells, lymphocytes, and fibroblasts, carry lipids, proteins, genetic materials, and transmit biological information in vivo[14-17]. Recent evidence indicated that EVs gained from KLF2-modified ECs (KLF2-EVs) attenuated the disease progression especially in pulmonary hypertension, atherosclerosis, myocardial ischemia-reperfusion(I/R) injury and other diseases associated with vascular remodeling[13, 16, 18].

Recently, more and more evidence indicated that monocyte-derived proinflammatory macrophages dominated the whole pathological process of dox-induced DCM and led to cardiac dysfunction[19]. KLF2-EVs was reported to inhibite the activation of Mo/Mø and delayed the progression of atherosclerosis[16]. Our previous work also found that KLF2- EVs attenuated myocardial I/R injury by inhibiting Ly6C\text{high} monocytes homing to the heart[13]. Based on these results, we established this study to explore the therapeutic potential of KLF2-EVs on the regulation of cardiac inflammation in the DCM.

**Methods**

**Animal experimental protocol**

This study of animal experiments was approved with the regulations and guidelines set by the Institutional Ethics Committee of Nanjing Drum Tower Hospital (Approval No. 2020AE01081). We purchased 8-week-old C57BL/6 male mice at weight of 20-24g, from Model Animal Research Center of Nanjing University. Animals received a standard laboratory diet and free access to food and water. They stayed in a controlled room temperature of 20°C to 25°C and humidity of 40% to 70%, with a 12-h light–dark cycle. These mice were randomly divided into the control and DCM group. The DCM group mice were established by intrapulmonary injection of doxorubicin (DOX) (20 mg/kg in total) dissolved in saline, whereas the control group mice were injected with an equal quantity saline. After DOX injection 7 days, the DCM group was randomly divided into DCM+KLF2-EVs group and DCM+PBS group, and then mice respectively received 200\mu l PBS and 200\mu l PBS containing 100\mu g KLF2-EVs through the tail vein.

**Exosome isolation and identification**

When the ECs grew to 70-80% confluency, the medium was changed to complete medium containing 5% EVs-depleted fetal bovine serum, and then continue to culture for 48 hours. The EVs were extracted by standard differential centrifugation. Cell culture supernatants were centrifuged at 3,000 g for 25 min and 10,000 g for 1 h at 4 °C to remove dead cells and debris. Later, supernatants were centrifuged at 100,000g for 3 h at 4 °C. Finally, the collected EVs were resuspended with phosphate-buffered saline PBS.

The morphology of exosomes was observed using transmission electron microscope (JEM-1011 Japan). The number and size were assessed using the NanoSight NS300 system (NanoSight, UK). The EVs were identified by marker proteins, Alix, TSG101, CD63 and CD9 using western blot. The total protein concentration of EVs was determined by BCA assay (Thermo Scientific) for measurement of total protein.
Statistical analysis

Statistical analysis was analyzed by GraphPad Prism 8.0 (Graph Pad Prism Software Inc., San Diego, CA, USA). For comparisons between multiple groups, the differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test. A p-value < 0.05 means the difference was statistically significant. Quantitative data are expressed as mean ± standard deviation (SD).

Results

The isolation and identification of KLF2- EVs

The EVs were extracted from the culture supernatants of KLF2-HUVECs through ultracentrifugation (Figure 1A). To identify the isolated vesicles were indeed EVs, we described their morphological and phenotypic characteristics[20]. First, transmission electron microscopy (TEM) showed that KLF2- EVs had double-layer membrane structure (Figure 1B). Then, the nanoparticle tracking analysis (NTA) displayed the diameters of the vesicles were within the range of 100-150 nm, with peak at 148 nm (Figure 1C). Lastly, western blot analysis confirmed that these vesicles expressed ALIX, TSG101, CD63, and CD9, which were recognized as specific membrane proteins for EVs (Figure 1D).

Isolation and characterization of KLF2-EVs. (A) EVs were extracted by serial differential centrifugation and ultracentrifugation. (B) The morphology of EVs was analyzed by TEM (scale bar, 100 nm). (C) The diameters of isolated EVs were demonstrated by NTA. (D) Representative images of western blot to evaluate the EVs typical protein markers, such as ALIX, CD9, CD63 and TSG101.

KLF2- EVs treatment improves left ventricular function in DCM

Mice received repetitive low-dose injections of DOX (20 mg/kg in total) and then followed by twice KLF2- EVs treatment (Figure 2A). Five weeks after the first DOX injection, echocardiogram was obtained (Figure 2 and Table S1 for detailed data). In DOX+PBS group, LVEF (Figure 2C) and LVFS (Figure 2D) were markedly reduced, meanwhile LVIDd (Figure 2E) and LVIDs (Figure 2F) were increased compared with control. This indicated that the animal model of DCM was constructed successfully. Treatment with KLF2- EVs improved LVEF (Figure 2C) and LVFS (Figure 2D), meanwhile decreased LVIDd (Figure 2E) and LVIDs (Figure 2F). To further explore the effect of KLF2-EVs on the cardiac structure, hearts were harvested and were stained with Masson trichrome (MT) and Hematoxylin and eosin (H.E.). Compared with the Control group, H.E. staining revealed that DCM + PBS group mice had larger left ventricular cavity and thinner myocardial thickness, and while KLF2- EVs treatment could attenuate this change (Figure 2G–2H). Further, MT staining showed the DCM+KLF2- EVs group mice had less myocardial interstitial fibrosis, compared with the DCM+PBS group mice(Figure 2I–2J). Therefore, these findings demonstrated that KLF2- EVs has a protective effect on cardiac function.

KLF2-EVs improves heart function and ameliorate ventricular remodeling.
Mice received repeated low-dose injections of DOX (20 mg/kg in total)) and then KLF2- EVs (100μg KLF2-EVs+150μL PBS) was administered two times via tail vein. Left ventricular function was assessed by echocardiography (representative short axis view of parasternal M-mode ultrasound). (C) Left ventricle ejection fraction (LVEF, %). (D) Left ventricle fractional shortening (LVFS, %). (E) Left ventricle end-diastolic diameter (LVIDd, mm). (F) Left ventricle end-systolic diameter (LVIDs, mm). (G-J) HE (G) and MT (I) were performed on heart sections taken 5 weeks after first DOX injection. Quantitative results of left ventricular area in HE stained sections of heart(I) and quantification of myocardial fibrosis of MT staining sections (J) were shown. Purple, Control; blue, DCM+PBS; green, DCM+KLF2-EVs. Mean ± SD; n ≥ 5. *, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.001.

KLF2-EVs reduced myocardial inflammation level of DCM

The pathogenesis of DCM is associated with chronic intramyocardial inflammation, characterized by increased circulating immune cells and inflammatory cytokines[21]. Therefore, we measured the mRNA levels of inflammatory factors in heart tissue by RT-qPCR. Compared with DOX+PBS group, KLF2-EVs treatment decreased the mRNA levels of pro-inflammatory cytokines including IL-1β and TNF-α (Figure3A), together increased the levels of anti-inflammatory cytokines such as IL-10 and TGF-β (Figure3B). Since macrophages are a key mediator in the dox-induced myocardial damage, we hypothesized that KLF2-EVs might modulate Mo/Mø responses. Flow cytometry analysis showed that Ly6C<sup>high</sup> Mo/Mø of cardiac tissues was increased in DCM+PBS group, however KLF2-EVs treatment remarkably reduced Ly6C<sup>high</sup> Mo/Mø (Figure3C-D). Besides, we analyzed the monocytes in peripheral blood. KLF2-EVs treatment moderately reduced Ly6C<sup>high</sup> monocytes, but there was no significant difference between DCM+PBS group and DCM+EVs group (Figure3E-F). The results presented that KLF2-EVs treatment downregulated Ly6C<sup>high</sup> Mo/Mø in heart tissue.

KLF2- EVs reduced the expression of Inflammatory factors level and decreased Ly6C<sup>high</sup> Mo/Mø in heart tissue. (A) RT-qPCR analyses of IL-1β and TNF-α. (B) RT-qPCR analyses of IL-10 and TGF-β. (C) Representative flow cytometry plots showed CD11b+Ly6C<sup>high</sup> in heart. (D) Quantification of CD11b+Ly6C<sup>high</sup> cells (Ly6C<sup>high</sup> Mo/Mø) within heart tissue. (E) Representative flow cytometry plots showed CD11b+Ly6C<sup>high</sup> in peripheral blood. (F) Quantification of CD11b+Ly6C<sup>high</sup> cells (Ly6C<sup>high</sup> Mo/Mø) within blood. Purple, Control; blue, DCM+PBS; green, DCM+KLF2-EVs. Mean ± SD; n=5. *, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.001.

KLF2-EVs prevented Ly6C<sup>high</sup> monocytes recruitment from bone marrow

We observed that Ly6C<sup>high</sup> Mo/Mø were decreased in the heart of KLF2-EVs-treated mice and the specific mechanism was not clear. Next, we focused on inflammatory response after KLF2-EVs treatment. The homing of immune cells, mainly monocytes, are mainly from the spleen and bone marrow into the heart after ischemic heart disease and myocarditis [22, 23]. First, we performed flow cytometry on the spleen tissue. There was no significant change of Ly6C<sup>high</sup> Mo/Mø between DCM+KLF2-EVs group and
DCM+PBS group (Figure 4A–B). It indicated that KLF2-EVs alleviating cardiac inflammation was not directly related to spleen. Next, we assumed that the bone marrow might be involved in the regulation of heart inflammation. We extracted the cells from bone marrow, and then analyzed Ly6C<sup>high</sup> monocytes (Figure 4C). We found that KLF2-EVs treatment increased the retention of Ly6C<sup>high</sup> monocytes in the bone marrow (Figure 4D). However, there was no significant difference of Ly6C<sup>low</sup> monocytes among the groups (Figure 4E). Taken together, these results suggested that KLF2-EVs suppressed DCM cardiac inflammatory cells by restraining the mobilization of Ly6C<sup>high</sup> monocytes from bone marrow.

Finally, we assessed the possible molecular mechanism of KLF2-EVs and Ly6C<sup>high</sup> Mo/Mø recruitment in bone marrow. Previous studies have found that the migration of mature Ly6C<sup>high</sup> monocytes was mediated by MCP-1 / CCR2 molecules[24, 25]. Here we extracted the cells from from bone marrow, and observed that the protein level of CCR2 was decreased in the DCM+KLF2-EVs group, compared with DCM+PBS treatment group (Figure 4F–G).

KLF2-EVs prevented Ly6C<sup>high</sup> monocytes recruitment from bone marrow by inhibiting the expression of CCR2. (A) Representative flow cytometry plots showed Ly6C<sup>high</sup> monocytes (CD11b+Ly6C<sup>high</sup>) in spleen. (B) Quantification of Ly6C<sup>high</sup> monocytes in spleen tissues. (C) Representative flow cytometry plots showed Ly6C<sup>high</sup> monocytes (CD11b+Ly6C<sup>high</sup>) and Ly6C<sup>low</sup> monocytes (CD11b+Ly6C<sup>low</sup>) in bone marrow. (D–E) Quantification of Ly6C<sup>high</sup> monocytes (D) and Ly6C<sup>low</sup> monocytes in bone marrow. (F) Representative Western blot images of CCR2. (G) Quantitative analysis of CCR2 protein levels in bone marrow. Representative images of western blot and quantification to assess expression of CCR2. Purple, Control; blue, DCM+PBS; green, DCM+KLF2-EVs. Mean ± SD. n=5. *, P<0.05; **, P<0.01; ***, P<0.001.

**Discussion**

The main finding of this study is that KLF2-EVs regulates the recruitment of cardiac Ly6C<sup>high</sup> Mo/Mø and improves cardiac function. This beneficial effect is related to inhibiting the mobilization of Ly6C<sup>high</sup> monocytes in bone marrow by targeting CCR2 protein expression. Our results suggest that KLF2-EVs may be a potential therapeutic target for the prevention and treatment of DCM.

As we all know, ECs are critical in vascular anti-inflammatory and anti-thrombosis[26, 27]. KLF2 is activated by shear stress and plays a key role in the development of lung[18, 28]. After birth, KLF2 is mainly expressed in ECs, inhibiting the activation of ECs under a variety of inflammatory stimuli[11]. EVs are biological vesicles with membrane structure secreted by cells, and they are essential mediators for intercellular information transmission and regulation of cell function[29-33].

Studies revealed that EVs secreted by ECs regulated the activation of monocytes or phenotype of macrophages[16, 31]. Instead, changes in EVs secretion might affect vascular inflammation in cardiovascular diseases. Recently, the development of pulmonary hypertension was associated with reduced KLF2 signaling, and KLF2-regulated EVs play an important regulatory role in vascular remodeling.
and vascular homeostasis[18]. Although the beneficial effect of KLF2-EVs has been identified in cardiovascular disease, the underlying functions and mechanisms in DCM have not been fully studied.

In this study, the mouse DCM model was established by intraperitoneal injection of DOX to simulate the formation of human DCM. We observed that KLF2-EVs treatment improved cardiac function and inhibited ventricular remodeling. Besides, inflammatory cells have an essential effect on the pathogenesis of DCM, especially macrophages. Previous studies have shown that macrophages were increased in human and mice DCM heart tissue[34, 35]. In DOX-induced cardiomyopathy, resident macrophages exhibited increased proliferation and conferred a reparative role, while monocyte-derived macrophages primarily exhibited a pro-inflammatory phenotype that dominated the whole DCM[19]. The EVs were derived from human umbilical vein endothelial cells in this experiment. Here, we found KLF2-EVs increased the level of anti-inflammatory factors and inhibited the level of pro-inflammatory factors. Previous studies also showed that exosomes derived from mouse coronary endothelial cell had anti-inflammatory effects[13]. Furthermore, KLF2-EVs reduced Ly6C^{high} Mo/Mø in myocardial tissue and peripheral blood, but there was no statistical difference in peripheral blood. The previous study found the heart function was significantly improved in doxorubicin-induced mouse DCM after removing monocyte-derived macrophages by clodronate liposomes[19]. So KLF2-EVs exerts cardioprotective effect by regulating the Mo/Mø system in DCM.

The cardiac resident Mo/Mø are relatively few, so most Mo/Mø are recruited from the periphery when inflammation occurs in myocardial tissue. Next, we speculated that the possible mechanism of down regulating myocardial Ly6C^{high} Mo/Mø is that KLF2-EVs inhibited the mobilization and recruitment of Ly6C^{high} Mo/Mø. Cardiac pro-inflammatory macrophages are mainly derived from the differentiation of peripheral monocytes. Recent evidence showed that the local collected Ly6C^{high} Mo/Mø in the heart were mainly from the spleen and bone marrow after myocardial I/R injury[36]. At the beginning of inflammation, Mo/Mø are mobilized from the spleen tissue[37]. Then the mature Ly6C^{high} monocytes mobilizing from bone marrow enter blood and then are recruited to heart. In this research, we found that KLF2-EVs treatment did not affect Ly6C^{high} Mo/Mø in spleen, but it increased the retention of Ly6C^{high} Mo/Mø in bone marrow. Mature Ly6C^{high} monocytes migrating from bone marrow to blood and from blood is mediated by MCP-1/CCR2[38]. The expression of CCR2 protein decreased after KLF2-EVs treatment, suggesting that KLF2-EVs effectively inhibited CCR2 expression. Taken together, our findings suggested that KLF2-EVs inhibited the mobilization of Ly6C^{high} Mo/Mø in the bone marrow by inhibiting CCR2 protein expression. This provides a potential approach for treating the DCM.

There are some limitations to our study. The pathogenesis of DCM is complicated and related to many factors[39, 40]. Thus, establishing an ideal animal model is crucial for studying the pathogenesis of DCM. We constructed the mouse DCM model by injecting DOX intraperitoneally. Dox damages cardiomyocytes and local fibrosis gradually replaces the injured myocardial tissue, which causes similar pathophysiological changes of DCM and subsequent congestive heart failure[41]. However, this method cannot fully simulate various forms of DCM. In addition, the main concentrating sites of EVs are the liver,
lungs, gastrointestinal tract, and spleen. We need to inject a great number of EVs to confirm enough EVs to exert its effect. Finally, there are multiple types of microRNAs in EVs, which microRNA plays a major role in the development of DCM remains further studied.

Conclusions

We show here that KLF2-transduced ECs-derived EVs improve cardiac systolic function and reduce ventricular remodeling in DOX induced DCM mice. This effect may be mediated by down regulating the expression of CCR2 protein, thereby inhibiting the mobilization of \( \text{ly6}^{\text{high}} \) monocytes in bone marrow. This study provided a new therapeutic approach for DCM.

Declarations

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Author information

Wenfeng Zhang, Ziwei Chen and Shuahua Qiao contributed equally to this work.

Authors’ contributions

BX, QL and WH designed the experiments. WZ, ZH and SQ performed the experiments, prepared the figures and wrote the manuscript. SC, HZ and XW provided technical support. All authors read and approved the final manuscript.

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Availability of data and materials

All data used to generate these results is available in the main text and supporting information.

Ethics approval and consent to participate
Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**
Isolation and characterization of KLF2-EVs. (A) EVs were extracted by serial differential centrifugation and ultracentrifugation. (B) The morphology of EVs was analyzed by TEM (scale bar, 100 nm). (C) The diameters of isolated EVs were demonstrated by NTA. (D) Representative images of western blot to evaluate the EVs typical protein markers, such as ALIX, CD9, CD63 and TSG101.
Figure 2

KLF2-EVs improves heart function and ameliorate ventricular remodeling. (A) Mice received repeated low-dose injections of DOX (20 mg/kg in total)) and then KLF2- EVs (100μg KLF2-EVs+150μL PBS) was administered two times via tail vein. (B) Left ventricular function was assessed by echocardiography (representative short axis view of parasternal M-mode ultrasound). (C) Left ventricle ejection fraction (LVEF, %). (D) Left ventricle fractional shortening (LVFS, %). (E) Left ventricle end-diastolic diameter
(LVIDd, mm). (F) Left ventricle end-systolic diameter (LVIDs, mm). (G-J) HE (G) and MT (I) were performed on heart sections taken 5 weeks after first DOX injection. Quantitative results of left ventricular area in HE stained sections of heart(I) and quantification of myocardial fibrosis of MT staining sections (J) were shown. Purple, Control; blue, DCM+PBS; green, DCM+KLF2-EVs. Mean ± SD. n ≥ 5. *, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.001.

Figure 3

KLF2-EVs reduced the expression of inflammatory factors level and decreased Ly6Chigh Mo/Mφ in heart tissue. (A) RT-qPCR analyses of IL-1β and TNF-α. (B) RT-qPCR analyses of IL-10 and TGF-β. (C)
Representative flow cytometry plots showed CD11b+Ly6Chigh in heart. (D) Quantification of CD11b+Ly6Chigh cells (Ly6Chigh Mo/Mø) within heart tissue. (E) Representative flow cytometry plots showed CD11b+Ly6Chigh in peripheral blood. (F) Quantification of CD11b+Ly6Chigh cells (Ly6Chigh Mo/Mø) within blood. Purple, Control; blue, DCM+PBS; green, DCM+KLF2-EVs. Mean ± SD. n=5. *, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.001.

**Figure 4**

KLF2-EVs prevented Ly6Chigh monocytes recruitment from bone marrow by inhibiting the expression of CCR2. (A) Representative flow cytometry plots showed Ly6Chigh monocytes (CD11b+Ly6Chigh) in spleen. (B) Quantification of Ly6Chigh monocytes in spleen tissues. (C) Representative flow cytometry
plots showed Ly6Chigh monocytes (CD11b+Ly6Chigh) and Ly6Clow monocytes (CD11b+Ly6Clow) in bone marrow. (D–E) Quantification of Ly6Chigh monocytes (D) and Ly6Clow monocytes in bone marrow. (F) Representative Western blot images of CCR2. (G) Quantitative analysis of CCR2 protein levels in bone marrow. Representative images of western blot and quantification to assess expression of CCR2. Purple, Control; blue, DCM+PBS; green, DCM+KLF2-EVs. Mean ± SD. n=5. *, P<0.05; **, P<0.01; ***, P<0.001.

**Supplementary Files**

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