**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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**SUPPLEMENTAL METHODS**

**Cell culture and transfection**

The human umbilical vein endothelial cells (HUVECs) were cultivated in ECs culture medium with 1% Penicillin/Streptomycin, 1% ECs growth supplement and 10% fetal bovine serum. Further, we constructed the GV358 vector carrying KLF2 cDNA, and co-transfected it with the lentiviral backbone plasmid into HEK293A cells to get the recombinant lentiviral vector Lv-KLF2. ECs were cultured overnight in a 6-well plate at a density of 1×10^6 cells/mL. Lentiviruses were diluted with complete medium containing HitransG P (GeneChem, China) and then added to ECs. After 12 hours of transfection at 37°C, we changed the medium with fresh virus-free medium. Continuous cultivation for 72 hours, green fluorescence (GFP positive) was detected by fluorescence microscope (ix53, Olympus, Japan). Next, to remove negative cells, puromycin (5μg/ml) was added to the culture medium and the survival cells were KLF2-transfected HUVECs (KLF2- HUVECs).

**Echocardiography**

5 weeks after the first DOX injection, mice were anesthetized with 1.5%–2% isoflurane in 100% oxygen. Echocardiography (Vevo®2100 Imaging System, Visualsonics) was performed from parasternal two-dimensional long and short axes. The index of left ventricular end‐diastolic diameter (LVIDd) and left ventricular end‐systolic diameter (LVIDs) were recorded. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated as described previously. (LVEF[%] = [ (LVIDd3 - LVIDs3 )/LVIDd3] × 100；LVFS (%) = [(LVIDd − LVIDs) / LVIDd] × 100）

**Histological analysis**

Histology of hearts was assessed 5 weeks after first doxorubicin injection. We collected the hearts in diastole late term with intraventricular injection of 10% potassium chloride (KCl).

The heart was fixed with 4% phosphate-buﬀered formalin (pH 7.4). Then the tissues were dehydrated by gradient ethanol and set in paraffin which cut into 5‐μm sections. Hematoxylin-eosin (H.E.) was applied to analyze the global heart morphology. and Masson trichrome (MT) to identify cardiac fibrosis. Quantification of cardiac left ventricular area and fibrosis was calculated by ImageJ.

**Flow cytometry analysis**

Isolated cell suspensions from heart, spleen, bone marrow and peripheral blood were obtained by gentle MACS™ Dissociator (Miltenyi Biotec). Then the samples were incubated with CD11b‐FITC (BD Bioscience) and Ly6C-PE (BD Bioscience) for 30 minutes at 4°C. Data from FACS Aria flow cytometer (BD Bioscience) were analyzed with FlowJo software. We used CD11b and Ly6C to describe monocyte phenotype subset and used percentage to evaluate Ly6Chigh and Ly6Clow monocyte/macrophages (Mo/Mø). For example, within heart samples, percentage was the Ly6Chigh Mo/Mø numbers to all living cardiac cells numbers, and within peripheral blood samples, percentage was the Ly6Chigh monocytes numbers to all blood living cells numbers.

**RNA extraction and reverse‐transcription quantitative polymerase chain reaction**

**(RT-qPCR)**

Total RNA was extracted from heart tissue following Trizol (Invitrogen) according to the manufacturer’s instructions. Then cDNA was synthesized by the HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme) and qPCR was carried out by ChamQ SYBR qPCR Master Mix (High ROX Premixed) (Vazyme). We used 2−ΔCt to calculate relative gene expression level and data were normalized to GAPDH expression. All the primers involved were as follows:

|  |  |
| --- | --- |
| Primer | sequence |
| GAPDH forward | TGTGTCCGTCGTGGATCTGA |
| GAPDH reverse | TTGCTGTTGAAGTCGCAGGAG |
| TNFα forward | CCCCAAAGGGATGAGAAGTTC |
| TNFα reverse | GCTTGTCACTCGAATTTTGAGAA |
| IL-1β forward | TGAAGTTGACGGACCCCAAA |
| IL-1β reverse | TGATGTGCTGCTGTGAGATT |
| TGFβ1 forward | GAAGGACCTGGGTTGGAAGTGGATC |
| TGFβ1 reverse | TGTGTTGGTTGTAGAGGGCAAGGAC |
| IL-10 forward | GTTGCCAAGCCTTATCGGGAA |
| IL-10 reverse | CCAGGGAATTCAAATGCTCCT |

**Western blot**

The total protein was extracted from bone marrow tissue and KLF2-EVs. Samples were lysed in cold lysis buffer with protease inhibitor (KEYGEN BIOTECH). 30µg of each protein sample was separated by SDS-PAGE gel (10%) and then transferred to PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% bovine serum albumin, the blots were probed with a primary antibody (1:1000) followed by a horseradish peroxidase-conjugated secondary antibody (1:5000). The following primary antibodies were used: CCR2 (Abcam, ab203128), GAPDH (MultiSciences, ab011), CD9 (Abcam, ab92726), CD63 (Abclonal, A5271), TSG101 (Proteintech, 28283-1-AP), Alix (Abcam, ab117600). The secondary antibody used were HRP-labeled Goat Anti-rabbit IgG(H+L) (Beyotime, A0208) and HRP-labeled Goat Anti-mouse IgG(H+L) (Beyotime, A0216). Enhanced chemiluminescence (ECL, thermofisher scientific) was used to detect the protein levels on the blots.

SUPPLEMENTAL TABLES

Supplemental Table 1 Echocardiographic parameters after KLF2-EVs treatment

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Control**  **(n=8)** | **DCM+PBS**  **(n=9)** | **DCM+KLF2- EXO**  **(n=9)** |
| **LVEF (%)** | 65.57±5.55 | 47.91±6.13\*\*\*\* | 59.52±8.17## |
| **LVFS (%)** | 35.49±1.38 | 23.90±3.65\*\*\*\* | 31.27±5.50## |
| **LVIDd (mm)** | 3.73±0.39 | 4.11±0.26\* | 3.73±0.26# |
| **LVIDs(mm)** | 2.42±0.38 | 3.12±0.28\*\*\* | 2.58±0.36## |
| **LVPWd(mm)** | 0.72±0.07 | 0.67±0.10 | 0.72±0.03 |
| **LVPWs(mm)** | 1.15±0.11 | 0.94±0.16\*\* | 1.11±0.10# |
| **IVSTd(mm)** | 0.73±0.06 | 0.71±0.07 | 0.73±0.04 |
| **IVSTs(mm)** | 1.21±0.12 | 1.03±0.06\*\* | 1.13±0.09 |
| **LV Vol;s (uL)** | 21.33±9.08 | 40.03±8.62\* | 24.80±8.32# |
| **LV Vol;d (uL)** | 60.24±11.34 | 76.52±12.66\*\*\* | 59.83±9.99## |
| **LV Mass (mg)** | 91.71±14.78 | 103.68±14.95 | 92.69±12.35 |
| **LV Mass**  **(Corrected)(mg)** | 73.37±7.97 | 82.94±11.96 | 74.15±9.88 |

LVEF, left ventricular ejection fraction; LVFS, left ventricle fractional shortening; LVIDs, left ventricle end-diastolic diameter ;LVIDs, left ventricle end-systolic diameter; LVPWd, left ventricular posterior wall thickness at end-diastole; LVPWs, left ventricular posterior wall thickness at end-systole; IVSTd, interventricular septal thickness at end-diastole; IVSTs, interventricular septal thickness at end-systole; LV Vol;d, left ventricle end diastolic volume; LV Vol;s, left ventricle end systolic volume; LV Mass, left ventricular heart weight ; LV Mass (Corrected): left ventricular heart weight(Corrected). \**P* < 0.05，\*\**P* < 0.01，\*\*\**P* < 0.001，\*\*\*\**P* < 0.0001 vs. Control。#*P* < 0.05，##*P* < 0.01 vs. DCM+PBS.