



# Interleukin-10 Deficiency Alters Endothelial Progenitor Cell-Derived Exosome Reparative Effect on Myocardial Repair via Integrin-Linked Kinase Enrichment

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**RATIONALE:** Systemic inflammation compromises the reparative properties of endothelial progenitor cell (EPC) and their exosomes on myocardial repair, although the underlying mechanism of loss of function of exosomes from inflamed EPCs is still obscure.

**OBJECTIVE:** To determine the mechanisms of IL-10 (interleukin-10) deficient-EPC-derived exosome dysfunction in myocardial repair and to investigate if modification of specific exosome cargo can rescue reparative activity.

**METHODS AND RESULTS:** Using IL-10 knockout mice mimicking systemic inflammation condition, we compared therapeutic effect and protein cargo of exosomes isolated from wild-type EPC and IL-10 knockout EPC. In a mouse model of myocardial infarction (MI), wild-type EPC-derived exosome treatment significantly improved left ventricle cardiac function, inhibited cell apoptosis, reduced MI scar size, and promoted post-MI neovascularization, whereas IL-10 knockout EPC-derived exosome treatment showed diminished and opposite effects. Mass spectrometry analysis revealed wild-type EPC-derived exosome and IL-10 knockout EPC-derived exosome contain different protein expression pattern. Among differentially expressed proteins, ILK (integrin-linked kinase) was highly enriched in both IL-10 knockout EPC-derived exosome as well as TNF $\alpha$  (tumor necrosis factor- $\alpha$ )-treated mouse cardiac endothelial cell-derived exosomes (TNF $\alpha$  inflamed mouse cardiac endothelial cell-derived exosome). ILK-enriched exosomes activated NF- $\kappa$ B (nuclear factor  $\kappa$ B) pathway and NF- $\kappa$ B-dependent gene transcription in recipient endothelial cells and this effect was partly attenuated through ILK knockdown in exosomes. Intriguingly, ILK knockdown in IL-10 knockout EPC-derived exosome significantly rescued their reparative dysfunction in myocardial repair, improved left ventricle cardiac function, reduced MI scar size, and enhanced post-MI neovascularization in MI mouse model.

**CONCLUSIONS:** IL-10 deficiency/inflammation alters EPC-derived exosome function, content and therapeutic effect on myocardial repair by upregulating ILK enrichment in exosomes, and ILK-mediated activation of NF- $\kappa$ B pathway in recipient cells, whereas ILK knockdown in exosomes attenuates NF- $\kappa$ B activation and reduces inflammatory response. Our study provides new understanding of how inflammation may alter stem cell-exosome-mediated cardiac repair and identifies ILK as a target kinase for improving progenitor cell exosome-based cardiac therapies.

**VISUAL OVERVIEW:** An online [visual overview](#) is available for this article.

**Key Words:** exosome ■ inflammation ■ interleukin-10 ■ myocardial infarction ■ stem cells

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## Novelty and Significance

### What Is Known?

- Bone marrow–derived endothelial progenitor cells (EPC) contribute to ischemic myocardial neovascularization.
- Systemic inflammation, such as loss of IL-10 (interleukin-10), leads to bone marrow–derived EPC dysfunction.
- Exosomes derived from bone marrow–derived EPC mimic parental cell reparative properties.

### What New Information Does This Article Contribute?

- Exosomes from IL-10 knockout mice (systemic inflammation) lose their reparative activity.
- Inflammation alters protein contents of EPC exosomes including enrichment of ILK (integrin-linked kinase).
- Exosomes enriched in ILK activate NF- $\kappa$ B (nuclear factor  $\kappa$ B) signaling in recipient cells and induce inflammatory gene expression.
- Small interfering RNA–mediated knockdown of ILK in IL-10 knockout EPCs rescue their cardiac repair ability.

Bone marrow stem cell therapies in clinical trials showed little or modest effect. It is known that preexisting comorbid conditions like systemic inflammation and diabetes mellitus in cardiac patients may compromise the functional activity of autologous stem/progenitor cells. Lately, exosomes or extracellular vesicles obtained from stem cells have been shown to mimic parental cell reparative activity in models of myocardial infarction. Whether exosomes obtained from stem cells from mice with systemic inflammation retain their reparative properties and whether inflammation alters protein cargo of exosomes is not known. Using IL-10 knockout mice a model of systemic inflammation, here we report that exosomes obtained from EPCs of these mice lose their angiogenic and cardiac reparative properties. We also show specific enrichment of ILK in these exosomes which induces NF- $\kappa$ B activation and NF- $\kappa$ B-mediated inflammatory gene activation in recipient cells. Finally, we report a strategy of ILK knockdown in exosomes to rescue inflammation-induced loss of their reparative activity.

## Nonstandard Abbreviations and Acronyms

<b>AMI</b>	acute myocardial infarction
<b>IL-10</b>	interleukin-10
<b>IL-10KO-EPC-Exo</b>	IL-10 knockout EPC-derived exosome
<b>ILK</b>	integrin-linked kinase
<b>ILK-KD-EPC-Exo</b>	IL-10 knockout EPC with ILK knockdown-derived exosome
<b>I<math>\kappa</math>B<math>\alpha</math></b>	inhibitor of $\kappa$ B $\alpha$
<b>MCEC</b>	mouse cardiac endothelial cell
<b>MCEC+TNF<math>\alpha</math>-Exo</b>	TNF $\alpha$ inflamed MCEC-derived exosome
<b>MCEC-Exo</b>	mouse cardiac endothelial cell-derived exosome
<b>miRs</b>	microRNAs
<b>NF-<math>\kappa</math>B</b>	nuclear factor kappa-light-chain-enhancer of activated B cells
<b>siRNA</b>	small interfering RNA
<b>TNF<math>\alpha</math></b>	tumor necrosis factor- $\alpha$
<b>WT-EPC-Exo</b>	wild-type EPC-derived exosome

showed promising yet modest results.<sup>3–5</sup> Besides poor survival and low integration of EPCs/stem cells in the myocardium, prolonged and unresolved inflammation post ischemic heart disease also significantly compromises EPC phenotype and may compromise their reparative function in myocardial repair.<sup>6,7</sup> Evidence suggests that almost one-fifth of patients after myocardial infarction (MI) are affected by complications with severe systemic inflammation as well as higher risk of death.<sup>8–10</sup> Systemic inflammation in patients is characterized with higher level of proinflammatory cytokine TNF $\alpha$  (tumor necrosis factor- $\alpha$ ) and lower level of anti-inflammatory cytokine IL (interleukin)-10 in the circulation. EPCs from these patients are dysfunctional with reduced cell survival, migratory, and angiogenic activities.<sup>11,12</sup> Understanding how inflammatory stimulus modulates EPC behavior is important for appropriate and timely containment and resolution of inflammation thereby enhancing EPC/progenitor cell–based myocardial repair and regeneration.

Recent studies suggest that stem cell–mediated myocardial repair is largely associated with paracrine effect.<sup>13,14</sup> Exosomes have recently been identified as major paracrine functional unit of stem cells. Exosomes are secreted from all types of cells and contain cell-specific small RNAs and proteins, cargo that largely determines exosome function.<sup>15</sup> Stem cell–derived exosomes show cardiac repair and regeneration properties akin to their parent cell themselves.<sup>15–17</sup> However, all exosomes, even when secreted by same cells, are not created equal; different pathophysiological conditions

**C**ardiovascular disease is the leading cause of morbidity and mortality worldwide, and stem cell therapy holds great promise for ischemic cardiac repair and regeneration.<sup>1,2</sup> Bone marrow–derived endothelial progenitor cell (EPC) progressed into clinical trials and

and stimuli of parental cells are known to alter exosome content and may modulate exosome function. Our previous studies using IL-10 knockout mice mimicking systemic inflammation conditions indicated that IL-10-deficient EPCs secrete exosomes that are deficient in promoting cell survival, migration, and angiogenesis *in vitro*, and their cargo shows differential protein and RNA expression pattern.<sup>18</sup> However, how systemic inflammation leads to EPC exosome dysfunction is not yet clear. Understanding the molecular mechanism of exosome function/dysfunction is urgent for exosome manipulation as well as application of this cell-free therapy in cardiovascular disease.

NF- $\kappa$ B (nuclear factor  $\kappa$ B) plays a key role in regulating inflammatory response. As a master transcription factor, after activation and nuclear translocation, it binds to DNA response elements and upregulates expression of many genes including inflammatory cytokines, chemokines, and adhesion molecules.<sup>19</sup> ILK (integrin-linked kinase) is an intracellular serine/threonine protein kinase and plays essential role in NF- $\kappa$ B activation.<sup>20,21</sup> ILK can be activated via either interaction between extracellular matrix with integrins or inflammatory stimulus (lipopolysaccharide, TNF $\alpha$ , etc) through the PI3K (phosphoinositide 3-kinase) pathway.<sup>22,23</sup> Activated ILK phosphorylates the p65 subunit of NF- $\kappa$ B, triggering NF- $\kappa$ B nuclear translocation and downstream gene expression. With the dissociation of p65, I $\kappa$ B $\alpha$  (inhibitor of  $\kappa$ B $\alpha$ ) is phosphorylated by IKK (I $\kappa$ B kinase), then rapidly ubiquitinated and degraded.<sup>24,25</sup> It has also been shown that ILK is not required for the inflammatory stimuli-induced classical NF- $\kappa$ B signaling pathway involving I $\kappa$ B $\alpha$  phosphorylation and degradation and nuclear translocation of NF- $\kappa$ Bp65 (p65 subunit of NF $\kappa$ B, RelA). Rather, ILK utilizes alternate mechanisms for NF- $\kappa$ B activation and transcription activity through direct p65 phosphorylation at Ser536 and is required for lipopolysaccharide-induced transactivation of p65 through Ser536<sup>23</sup>.

ILK is widely distributed in mammalian tissues, but its highest expression in heart suggests an essential role in cardiac function regulation.<sup>26</sup> As upstream molecule of the Akt (protein kinase B) and GSK3 $\beta$  (glycogen synthase kinase-3 beta) pathway, it acts in cell survival and proliferation,<sup>27</sup> but in cardiomyocytes, it appears to be prohypertrophic,<sup>28</sup> profibrosis,<sup>29</sup> proinflammation,<sup>30</sup> and it is induced during cardiac aging.<sup>31</sup> In the present study, we found ILK was highly enriched in both IL-10 knockout EPC (IL-10KO-EPC)-derived exosomes and TNF $\alpha$ -treated mouse cardiac endothelial cell (MCEC)-derived exosomes, suggesting its important role in exosome-mediated inflammatory response. Our study explored the role of exosome delivered ILK in NF- $\kappa$ B activation in recipient cell and how ILK-enriched exosomes involved in inflammatory response and whether ILK knockdown in exosomes may rescue myocardial repair ability of inflamed EPC-derived exosomes.

Data from our studies identify an essential role of ILK in EPC-exosome-mediated inflammatory response

via the activation of NF- $\kappa$ B pathway. We further report that knockdown of ILK in inflamed exosomes attenuates the ILK-enriched-exosome-mediated inflammatory response, inhibits the NF- $\kappa$ B pathway activation, and enhances the EPC-derived exosomes reparative activity in ischemic heart.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Expanded methods are included in [Online Data Supplement](#).

### Vertebrate Animals and Reagents

All experiments conform to the protocols approved by Temple University Institutional Animal Care and Use Committee. Eight-to-10-week-old wild-type (C57BL/6J, WT) and IL-10 knockout (B6.129P2-*Il10*<sup>mlC<sup>gn</sup>/J</sup>, IL-10KO) male mice were procured from Jackson Research Laboratory (Bar Harbor, ME) for EPC isolation. Ten-to-12-week-old WT (C57BL/6J, WT) male mice were procured from Jackson Research Laboratory (Bar Harbor, ME) for MI surgery. Studies were only performed in male mice to eliminate the possible confounding effects of reproductive hormones. Lipopolysaccharide was obtained from Sigma Aldrich Inc (St. Louis, MO). Recombinant Mouse TNF-alpha (aa 80-235) protein was obtained from R&D Systems Inc.

### Bone Marrow-Derived Endothelial Progenitor Cells Isolation, Culture, and Supernatant Collection

EPC isolation, *ex vivo* expansion, and culture of EPCs were performed as previously described.<sup>32</sup> For exosome studies, all FBS used in cell culture was Exosome-depleted FBS (ThermoFisher Scientific A2720801). Supernatant of WT-EPC and IL-10KO-EPC group was collected once daily from day 7 to day 10 for exosome isolation; for IL-10 knockout EPC with ILK knockdown-derived exosome (ILK-KD-EPC-Exo) group, small interfering RNA (siRNA) transduction was administrated onetime on day 7 IL-10KO-EPCs, and supernatant was collected onetime daily from day 8 to day 10 for exosome isolation.

### MCEC Cell Culture, Treatment, and Supernatant Collection

Immortalized MCEC line was purchased from CEDARLANE (CLU510) and maintained according to the manufacturer's instructions. Cells were cultured in DMEM with 10 mmol/L penicillin/streptomycin (Gibco), 10 mmol/L HEPES (Sigma), and 5% exosome-depleted-FBS (Thermo Fisher Scientific) and plated on cell culture dishes coated with 0.2% Gelatin (Sigma). For MCEC-derived exosome (MCEC-Exo) group, supernatant was collected onetime daily; for TNF $\alpha$  inflamed MCEC-derived exosome (MCEC+TNF $\alpha$ -Exo) group, MCECs were treated with TNF $\alpha$  100 ng/mL for 24 hours, washed with PBS, replenished with fresh medium, and supernatant collected after 24 hours; for TNF $\alpha$  inflamed MCEC with ILK knockdown-derived exosome group, MCECs were first treated with 100 ng/mL TNF $\alpha$  for 24 hours, washed with PBS, replenished with fresh medium,

then treated with siRNA for another 24 hours, and supernatant was collected after 24, 48, and 72 hours for further studies. For experiments involving MCECs with exosome treatment, exosome number was 100-fold of cell number.

### Exosome Isolation and Quantification

Exosome isolation, purification, storage, and characterization were performed as previously described.<sup>33</sup> Exosome protein level was quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher) as per manufacturer's instructions. Exosome particle number was quantified by NanoSight using Nanoparticle Tracking Analysis software (Malvern Panalytical).

### Exosome Mass Spectrometry Analysis

WT-EPC and IL-10KO-EPC-derived exosomes (50 µg protein) in PBS (duplicates in each group) were sent to Quantitative Proteomics Resource Core at University of Pennsylvania for mass spectrometry analysis as previously described.<sup>18</sup>

### siRNA Knockdown Experiment

siRNA against mouse ILK (sc-35667; Santa Cruz) or a negative control were used for siRNA knockdown experiments. Twenty-four hours after plating, cells were transfected with 100 nmol/L siRNA and Lipofectamine RNAiMax (Invitrogen) in OptiMEM (Invitrogen) media overnight. Cell and exosomes samples of 24, 48, and 72 hours after siRNA transfection were collected for ILK knockdown efficiency tests.

### Preparation of Cell Lysates, Exosome Lysates, and Western Blotting

Cells and exosomes lysates preparation and Western blotting were conducted as previously described.<sup>18,34</sup> As for antibody usage, beta-actin (sc-47778) was used for cell loading control, whereas flotillin-1 (sc-74566) and CD63 (sc-15363) were used for exosome loading control. Other involved antibodies include ILK (ab52480; Abcam), NF-κB p65 (No. 6956S; CST), NF-κB-Phospho-p65 (No. 3033; CST), IκBα (No. 9242; CST), Phospho-IκBα (No. 9246; CST). All antibody information can be found in Online Table III.

### Tube Formation Assay

$4 \times 10^4$  MCECs were plated on 130 µL Matrigel (Corning) per well in a 48-well plate and simultaneously treated with MCEC-Exo ( $4 \times 10^6$ ), MCEC+TNFα-Exo ( $4 \times 10^6$ ), TNFα inflamed MCEC with ILK knockdown-derived exosome ( $4 \times 10^6$ ), or PBS. After incubation at 37°C in an atmosphere of 5% CO<sub>2</sub> for 6 hours, gels were observed by using a phase-contrast microscope. The total branch points were counted in each well. Results are represented as mean±SEM for 3 independent experiments.

### Induction of Acute MI

Mice underwent surgery to ligate the left anterior descending coronary artery as reported previously<sup>35</sup> followed by administration of exosomes (WT EPC-derived exosome [WT-EPC-Exo], IL-10 knockout EPC-derived exosome [IL-10KO-EPC-Exo], ILK-KD-EPC-Exo all  $2 \times 10^9$  particles per group) or PBS intramyocardially into the left ventricular wall (border zone) at 3 different

locations immediately after left anterior descending ligation. The sham group underwent the same open chest procedure without ligation (10–13 mice/group). Heart tissue was harvested on day 5 or around a month (day 30 or day 31) after AMI for histological analysis. Whenever possible, experiments were performed in a blinded fashion. For example, experiments were blinded for the surgeon performing MI or sham surgeries. Animals were excluded only under circumstances that there was surgery associated mortality (<24 hours post-surgery) or mice with no-MI, otherwise there were no exclusions in this study. Heart tissue was harvested on day 5 or around a month (day 30 or day 31) after AMI for histological analysis, MI area percentage in whole heart data was collected for Trichrome analysis, border zone data was collected for vessel density and TUNEL (terminal deoxynucleotidyl transferase) analysis, data from all slides were used for calculation, and representative slides were shown in figures. Animal number calculations followed our published results of prior studies from our laboratory, which measured recovery from MI in these model systems. At a 2-sided significance of 0.05 and a power of 0.90, we estimated that 8 to 10 mice in each treatment group at each time point will be required.

### Echocardiography

Transthoracic 2-dimensional M-mode echocardiography was performed using the Vevo2100 (VisualSonics, Toronto, ON, Canada) equipped with a 30-MHz transducer as described previously.<sup>35</sup>

### Tissue Preparation, TUNEL Assay, CD31 Staining, and Masson Trichrome Staining

Heart tissue preparation, TUNEL assay (Roche), CD31 staining (R&D, AF3628), and Masson trichrome staining (Sigma) were performed as previously described.<sup>35,36</sup>

### Statistics

Results are expressed as the mean±SEM, computed from separate experiments. Comparison of ≥2 groups was performed by 1-way or 2-way ANOVA with Tukey multiple comparisons test. Significance for Figure 2D was calculated using unpaired Student *t* test using nonparametric measures and Mann-Whitney normality test.  $P < 0.05$  is considered statistically significant. Error bars represent ±SEM. Statistical analysis was performed using Graph Pad Prism v 7.0 software. The images presented in the article are randomly selected representative images of original data from multiple experiments and used as a visual representation of cumulative data.

## RESULTS

### Exosomes From IL-10KO EPCs Are Functionally Inert

To assess EPC-derived exosome dysfunction under IL-10 deficiency, WT-EPC-Exo, IL-10KO-EPC-Exo, or PBS were intramyocardially administered in AMI mouse model. Exosome size and yield was similar in WT and IL10 KO EPCs, suggesting loss of IL-10 gene does not alter

exosome secretion (Online Figure I). Left ventricle cardiac function was measured with echocardiography serially up to a month. Subgroups of mice were euthanized on day 5 post-MI for cardiac apoptosis analysis. Masson trichrome staining and CD31 staining on day 31 post-MI heart samples were used for cardiac remodeling and angiogenesis analysis. Our data revealed that compared with saline, WT-EPC-Exo treatment significantly improved left ventricle cardiac function as evident from increased percentage ejection fraction and fractional shortening on day 31 post-MI (Figure 1A and 1B) and significant reduction in cardiomyocyte apoptosis as shown on day 5 post-MI heart samples (Figure 1C and 1D). Histological analysis on day 31 post-MI heart samples indicated that WT-EPC-Exo treatment significantly decreased MI scar size and promoted neovascularization (Figure 1E through 1H and Online Figure III). However, no cardiac beneficial effect was observed in IL-10KO-EPC-Exo-treated groups. Conversely, IL-10KO-EPC-Exo treatment enhanced apoptosis and inhibited post-MI angiogenesis. Taken together, these findings demonstrated that compared with WT-EPCs exosomes which showed reparative activities, exosomes from IL-10 deficient EPCs lose their myocardial repair, angiogenic, and cell survival activities.

### IL-10 Deficiency Altered EPC Exosome Protein Contents With Enrichment of ILK

To understand if differential protein cargo between exosomes might be involved in apparently opposing biological activities, mass spectrometry was conducted to evaluate the protein content of WT-EPC-Exo and IL-10KO-EPC-Exo. Protein expression pattern in 2 exosomes was significantly different with alteration in proteins related to multiple cellular functions/pathways (Online Table I). Proteins that were highly overexpressed in IL-10KO-EPC-Exo were categorized into biological process using gene ontology, and as expected, we found enrichment of inflammation-related proteins in IL-10KO-EPC-Exo compared with WT-EPC-Exo. We performed Western blotting to further validate protein expression from proteomic analysis. To establish that enriched proteins in IL-10KO EPC exosomes are induced due to inflammation and not by loss of IL-10 itself, we used exosomes from MCEC treated with TNF $\alpha$  as a surrogate for inflammatory model *in vitro*. Our results suggested IL-10 deficiency altered EPC-derived exosome protein content (Figure 2A). Several protein kinases showed differential expression (Online Figure II). We particularly focused on ILK as its role is well documented in inflammation, and it was highly enriched in IL-10KO-EPC exosomes (Figure 2B and Online Figure II). Western blotting confirmed that there was about 2- to 4-fold increase in ILK expression in both IL-10KO-EPC-Exo and MCEC+TNF $\alpha$ -Exo, without much difference in total cell protein content (Figure 2C and 2D). These data indicated that inflammatory stimulus

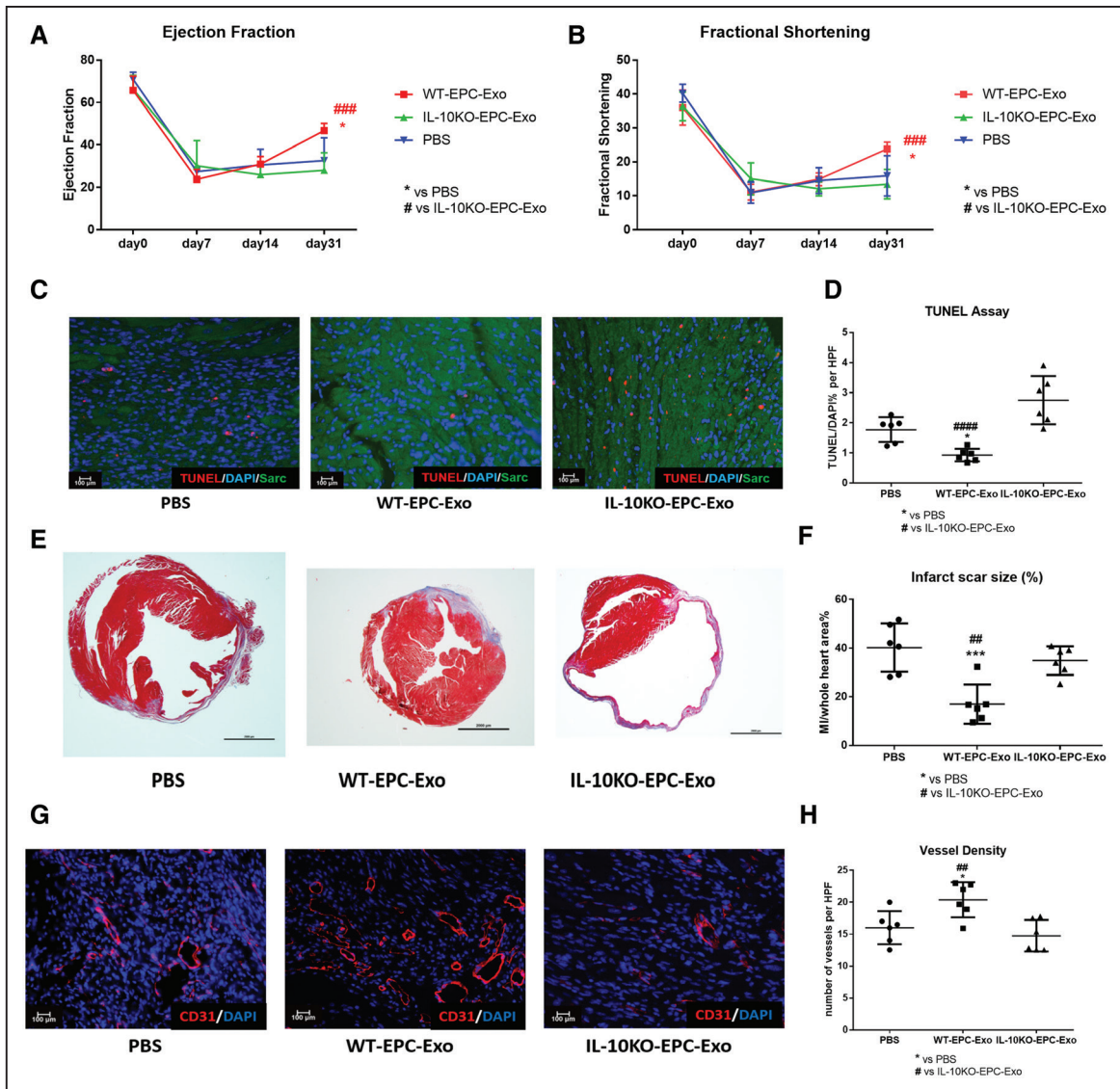
changed cell-derived exosome protein content, and ILK was generally enriched in inflamed exosomes, suggesting its potential role in mediating inflammatory response.

### ILK-Enriched Exosomes Activate the NF- $\kappa$ B Pathway in Recipient Cells

To elucidate the role of ILK-enriched exosomes in mediating the inflammatory response, MCECs were treated with MCEC-Exo, MCEC+TNF $\alpha$ -Exo, PBS, or TNF $\alpha$ . In both short-time (15 minutes, 30 minutes, 1 hour) and long-time (24 hours) treatment, MCEC+TNF $\alpha$ -Exo activated nuclear translocation of NF- $\kappa$ B (Figure 3A and 3B) and induced I $\kappa$ B $\alpha$  degradation (Figure 4A and 4B); 24-hour treatment triggered NF- $\kappa$ B downstream gene expression (Figure 4C). Similar results were observed in TNF $\alpha$ -treated group which was designed as positive control. No significant difference was observed between MCEC-Exo and PBS groups. Additionally, TNF-treated MCEC-Exo did not alter I $\kappa$ B $\alpha$  phosphorylation, suggesting an alternate mechanism of ILK-mediated NF $\kappa$ B activation. ILK has been shown to act via alternate NF $\kappa$ B activation by directly phosphorylating serine 536 residue of p65 subunit of NF $\kappa$ B and facilitating its nuclear translocation. Indeed, MCEC+TNF-Exo treatment led to efficient serine536 phosphorylation and nuclear translocation of p65 (Online Figure IV). Collectively, these results indicated that ILK-enriched exosomes activate NF- $\kappa$ B pathway in recipient cells and enhanced inflammatory response by enhancing inflammatory gene expression.

### ILK Knockdown Exosomes Showed Attenuated NF- $\kappa$ B Activation Effect

To confirm the role of ILK-enriched exosomes in activating inflammatory response in recipient cells, siRNA strategy was used for ILK knockdown in cells before the isolation of exosomes. siRNA-mediated ILK knockdown efficiency was  $\approx$ 70% to 90% in both EPCs and MCECs and their exosomes, as confirmed by Western blots (Online Figure V). To rule out the possibility that siRNA may transfer to exosomes and directly alter ILK expression, MCECs were treated with ILK KD (ILK-knock down) exosomes for 24 hours. No changes in ILK mRNA was detected (Online Figure VI). Repeat experiments with ILK-KD exosomes indicated that short-time exposure (15 minutes, 30 minutes, 1 hour) significantly attenuated NF- $\kappa$ B nuclear translocation, although some degree of NF- $\kappa$ B translocation was evident with 24-hour treatment at levels lower than TNF $\alpha$ -treated cell exosomes (Figure 5A and 5B). TNF $\alpha$  inflamed MCEC with ILK knockdown-derived exosome short-time (15 minutes, 30 minutes, 1 hour) treatment did not induce I $\kappa$ B $\alpha$  degradation (Figure 6A and 6B), and 24-hour treatment showed lower level of NF- $\kappa$ B-induced downstream gene expression (Figure 6C). Taken together, these observations suggest ILK-enriched exosomes activate inflammatory



**Figure 1. Interleukin-10 knockout endothelial progenitor cell–derived exosomes (IL-10KO-EPC-Exo) do not improve post-myocardial infarction (MI) cardiac repair.**

**A** and **B**, Echocardiographic analysis at various time points after MI. Wild-type EPC-derived exosome (WT-EPC-Exo)–treated mice showed significant improvement in % ejection fraction and % fractional shortening. IL-10KO-EPC-Exo–treated mice did not show any improvements in cardiac function (N=10–13/group;  $P=0.0103$ ,  $####P<0.0003$  in **A**;  $P=0.0105$ ,  $####P=0.0004$  in **B**). **C** and **D**, TUNEL (terminal deoxynucleotidyl transferase) analysis on day 5 heart samples for post-MI cell apoptosis measurement showed enhanced apoptotic cells in IL-10KO-EPC-Exo–treated mice (scale bar: 100  $\mu$ m, N=6,  $P=0.0367$ ,  $####P<0.0001$ ). **E** and **F**, Masson trichrome staining on day 31 heart samples for post-MI scar size measurement showed larger infarct size in IL-10KO-EPC-Exo group (scale bar: 2000  $\mu$ m, N=6,  $##P=0.0046$ ,  $***P=0.0005$ ). **G** and **H**, CD31 staining on day 31 heart for post-MI angiogenesis measurement (scale bar: 100  $\mu$ m, N=6,  $*P=0.0281$ ,  $##P=0.0053$ ; \*vs PBS group, #vs IL-10KO-EPC-Exo group). DAPI indicates 4'6-diamidino-2-phenylindole.

response in recipient cells through NF- $\kappa$ B activation. Blocking ILK in exosomes successfully inhibited NF- $\kappa$ B activation and inflammatory response in recipient cells.

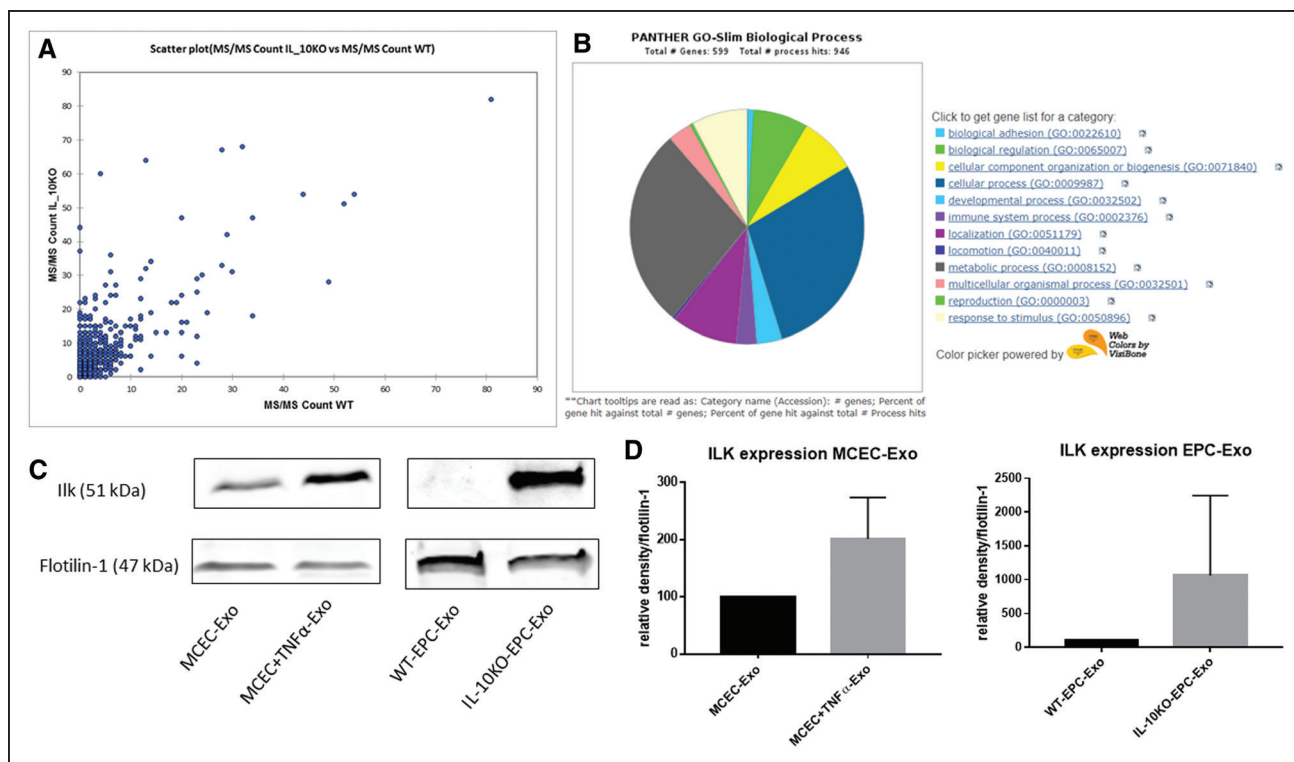
### ILK Knockdown in MCEC+TNF $\alpha$ -Exo Rescued Their Angiogenic Activity In Vitro

We have previously shown that IL-10KO-EPC-Exo inhibited tube formation ability of endothelial cells in vitro.<sup>18</sup> Similar inhibition in EC tubulogenesis was observed with the MCEC+TNF $\alpha$ -Exo treatment. On the contrary, TNF $\alpha$

inflamed MCEC with ILK knockdown-derived exosome treatment significantly enhanced tube formation and angiogenic capabilities of endothelial cells (Figure 7A and 7B). Therefore, the inhibiting effect on angiogenesis induced by inflamed exosomes was attenuated through ILK knockdown.

### ILK Knockdown in IL-10KO-EPC-Exo Rescued Their Reparative Activity, In Vivo

To further investigate ILK knockdown exosomes function in ischemic cardiac repair, we conducted same



**Figure 2.** ILK (integrin-linked kinase) enrichment in both IL-10 knockout endothelial progenitor cell–derived exosome (IL-10KO-EPC-Exo) and TNF $\alpha$  (tumor necrosis factor  $\alpha$ ) inflamed mouse cardiac endothelial cell–derived exosome (MCEC+TNF $\alpha$ -Exo).

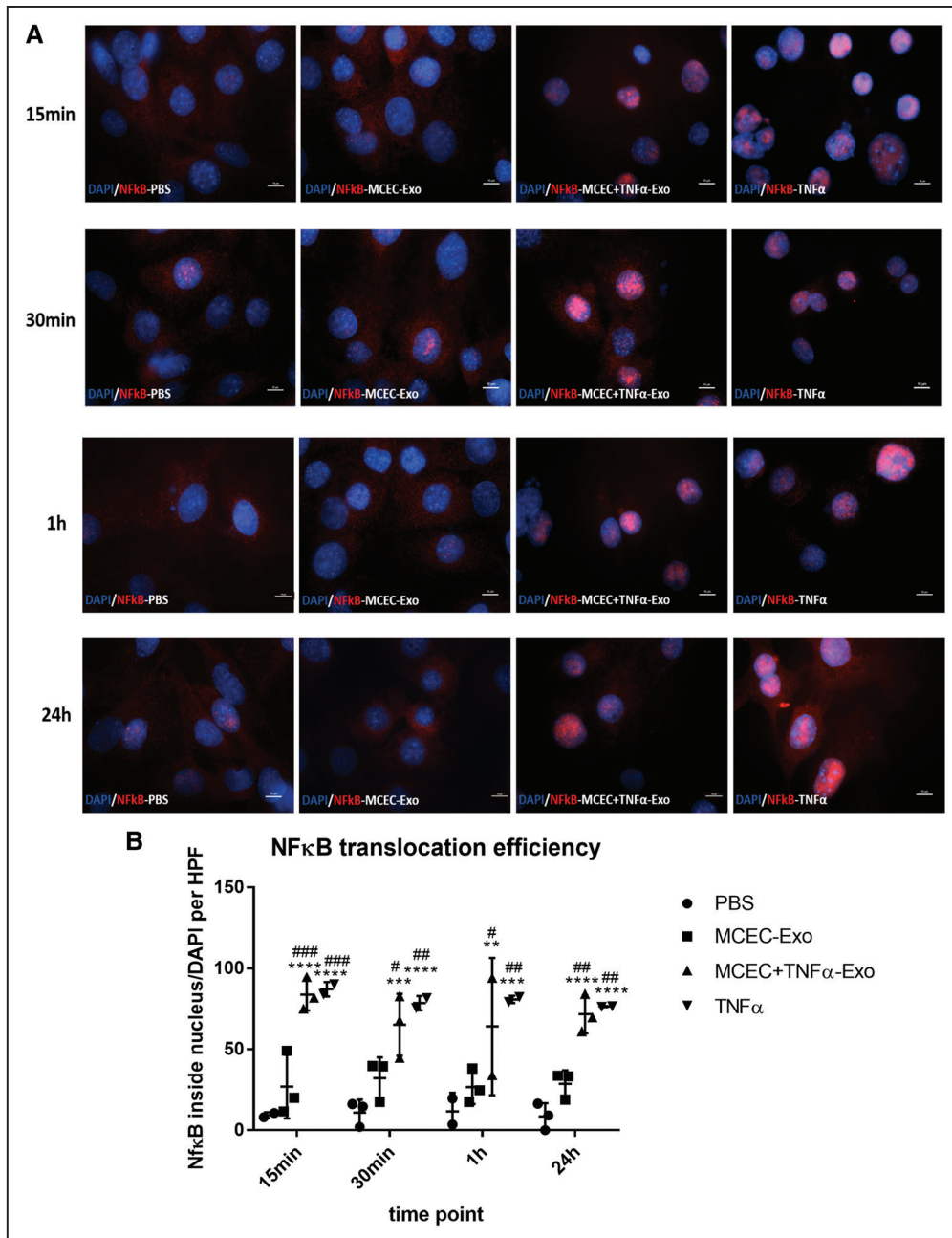
**A**, Scatter plot of mass spectrometry analysis showed different protein expression patterns in wild-type EPC-derived exosome (WT-EPC-Exo) and IL-10KO-EPC-Exo. **B**, Gene ontology analysis on proteins overexpressed in IL-10KO-EPC-Exo for biological process identification. **C** and **D**, Western blotting analysis verified ILK enrichment in both IL-10KO-EPC-Exo and MCEC+TNF $\alpha$ -Exo (\*vs MCEC-derived exosome [MCEC-Exo], N=5, \* $P=0.0368$ , #vs WT-EPC-Exo, N=3, ## $P=0.0087$ ). KO indicates knock out; and MS, mass spectrometry.

siRNA strategy on IL-10KO-EPC (Online Figure V) and collected ILK-KD-EPC-Exo for in vivo treatment in mouse AMI model and directly compared the reparative properties of IL-10KO-EPC-Exo with ILK-KD exo from IL-10KO-EPCs. Echocardiography, Masson trichome staining, and CD31 staining were performed as previously described. We observed significantly increased % ejection fraction and % fractional shortening in both WT-EPC-Exo–treated and ILK-KD-EPC-Exo–treated groups on day 30 post-MI, whereas IL-10EPC-Exo remained nonfunctional as shown earlier in Figure 1. (Figure 8A and 8B), suggesting improved left ventricle cardiac function. Histological analysis on day 30 post-MI heart samples showed significantly reduced scar size and increased capillary density in both WT-EPC-Exo and ILK-KD-EPC-Exo groups (Figure 8C through 8F), indicating ameliorated post-MI scar formation and enhanced neovascularization. Taken together, these data suggested ILK knockdown successfully rescued IL-10 deficiency/inflammation–induced EPC exosomes dysfunction in myocardial repair.

## DISCUSSION

Over the past 2 decades, stem cell–based therapy has emerged as promising therapeutic approach for ischemic

cardiac repair and regeneration.<sup>37</sup> Endothelial progenitor cells generated from bone marrow or peripheral blood have been applied in clinical studies. However, prolonged and unresolved inflammation after MI appears to worsen cardiac function recovery and may alter autologous progenitor cell biology thereby compromising the therapeutic effects of EPC transplantation.<sup>7</sup> IL-10 is a naturally occurring anti-inflammatory cytokine and has been shown to be involved in proliferation, antiapoptotic, and cell survival activities, dampens the inflammatory effects in overwhelming infections, and attenuate tissue damage. Multiple studies from our and other labs have established a protective role of IL-10 in cardiac injury and repair. After MI, IL-10–deficient mice have increased infarct size and heavier myocardial necrosis with more neutrophil infiltration. IL-10 treatment in MI mouse reduces inflammation, improves left ventricle cardiac physiology, stimulates M2 macrophage polarization and fibroblast activation, and improves cardiac remodeling.<sup>38</sup> However, human studies are not very consistent. Some studies find that in patients with acute MI, higher serum IL-10 within 24 hours after angioplasty is associated with reduced incidence of heart failure progression, whereas others show that higher IL-10 predicts recurrent cardiac events or heart failure.<sup>39,40</sup> In particular to stem cell therapy in ischemic heart disease, many studies from our lab



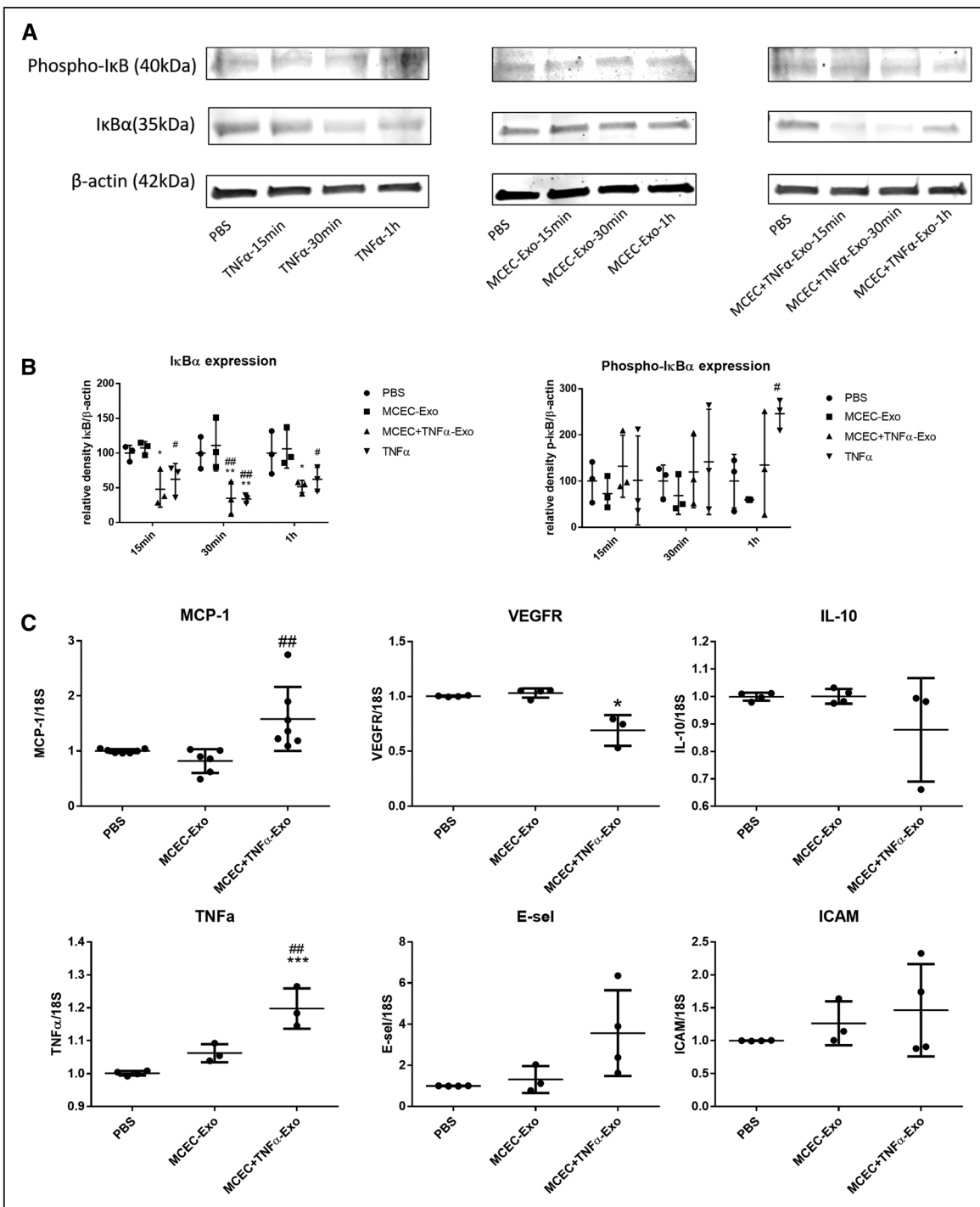
**Figure 3. TNF $\alpha$  (tumor necrosis factor  $\alpha$ ) inflamed mouse cardiac endothelial cell-derived exosome (MCEC+TNF $\alpha$ -Exo) activated NF- $\kappa$ B (nuclear factor  $\kappa$ B) nuclear translocation.**

**A** and **B**, NF- $\kappa$ B staining analysis showed both MCEC+TNF $\alpha$ -Exo and TNF $\alpha$  treatment triggered NF- $\kappa$ B nuclear translocation in recipient MCEC cells. \*vs PBS group (N=3, \*\* $P=0.0050$ , \*\*\* $P=0.0004$ , \*\*\*\* $P<0.0001$ ); #vs MCEC-derived exosome (MCEC-Exo) group (N=3, # $P=0.0380$ , ## $P=0.0066$ , ### $P=0.0004$ ; scale bar:10  $\mu$ m). DAPI indicates 4'6-diamidino-2-phenylindole; and HPF, high power field.

have shown that IL-10 deficiency impairs bone marrow-derived EPC survival and function in ischemic myocardium and IL-10 treatment improves cardiac remodeling and function, inhibits fibroblast progenitor cell migration and transdifferentiation, decreases cardiac fibrosis.<sup>35,36</sup> Our published studies also established that EPCs were functionally impaired under inflammatory stimulus.<sup>35</sup> IL-10KO mice mimicking systemic inflammation have high level of circulating TNF $\alpha$  and do not express IL-10 in circulation, thus mimicking the pathological condition of

systemic inflammation. Using IL-10KO mice as a model, we reported that IL-10KO-EPC-derived exosomes were functionally impaired in promoting cell survival, migration, and angiogenesis in vitro.<sup>18</sup> In this study, we further explored the effect of IL-10 deficiency on EPC-derived exosome content and function and on mechanistic basis of inflammation-induced stem cell-exosome dysfunction both in vitro and in vivo.

Recent studies revealed that functional benefits of stem cell therapy are largely mediated via paracrine effects.<sup>41-43</sup>

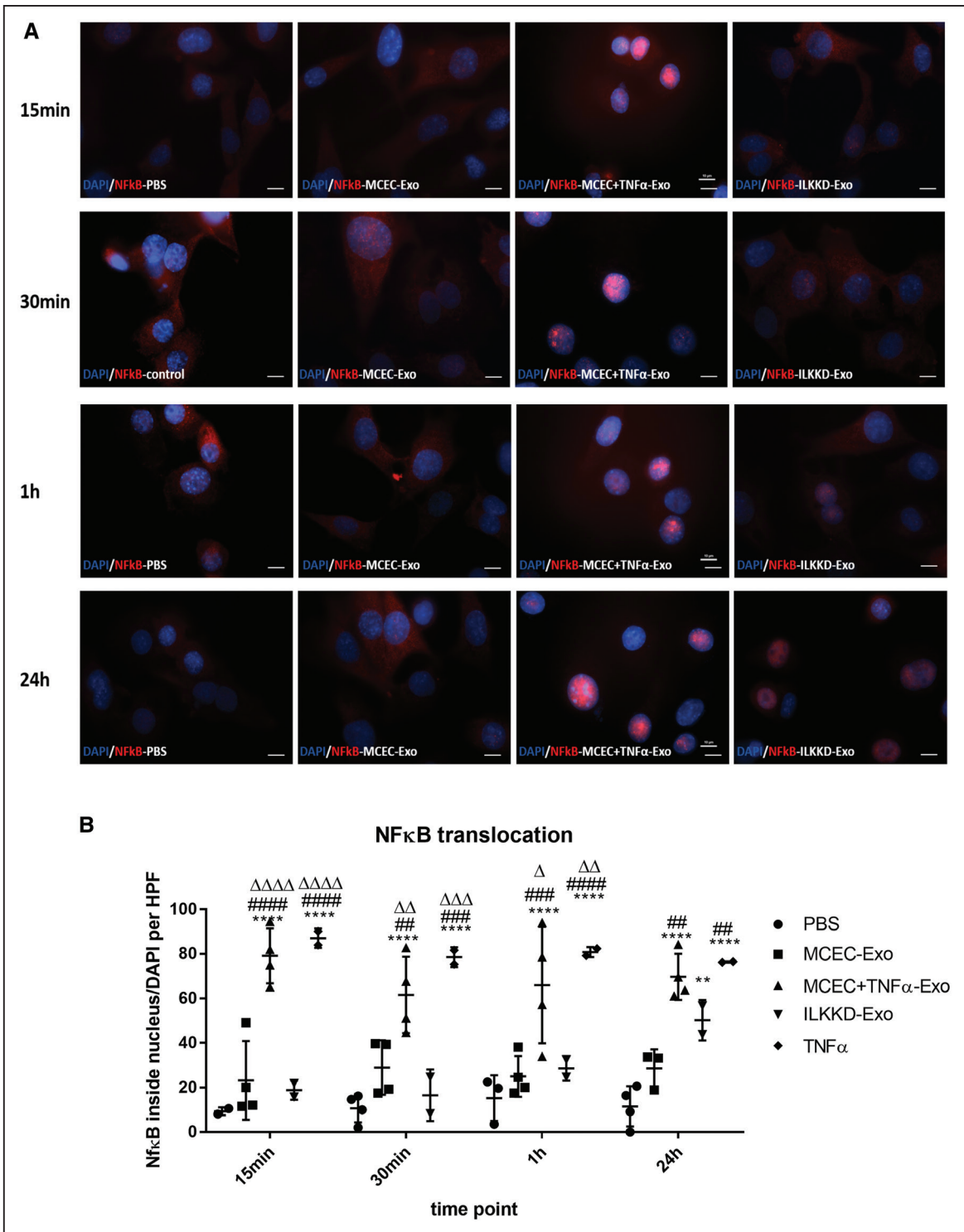


**Figure 4. TNFα (tumor necrosis factor α) inflamed mouse cardiac endothelial cell-derived exosome (MCEC+TNFα-Exo) activated NF-κB (nuclear factor κB) signaling pathway.**

**A** and **B**, Western blotting showed both MCEC+TNFα-Exo and TNFα activated NF-κB signaling pathway, decreased IκBα (inhibitor of κBα) expression in recipient MCEC cells (N=3, \*P=0.0399, \*\*P=0.0066, #P=0.0308, ##P=0.0018). **C**, Quantitative real-time polymerase chain reaction analysis showed 24 h of MCEC+TNFα-Exo treatment triggered NF-κB downstream gene expression in recipient MCEC cells (N=4, \*P=0.0250, \*\*\*P=0.0009); vs PBS group #vs MCEC-derived exosome (MCEC-Exo) group (##P=0.0047). E-sel indicates E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemoattractant protein; pIκBα, phosphorylated IκBα; and VEGFR, vascular endothelial growth factor receptor.

Exosomes have emerged as major paracrine functional units; they are involved in intercellular communication and are characterized with cell-specific cargo of proteins, small RNAs, and lipids. Large number of recent studies have explored cardiac repair and regeneration benefit of exosomes from

induced pluripotent stem cells,<sup>17</sup> mesenchymal stem cells<sup>44</sup> and bone marrow cells<sup>18</sup> in MI,<sup>45</sup> heart failure,<sup>46</sup> ischemia/reperfusion injury,<sup>47</sup> and many other pathologies. However, exosomes are not created equal and their cargo is actively under alteration reflecting the physiological or pathological

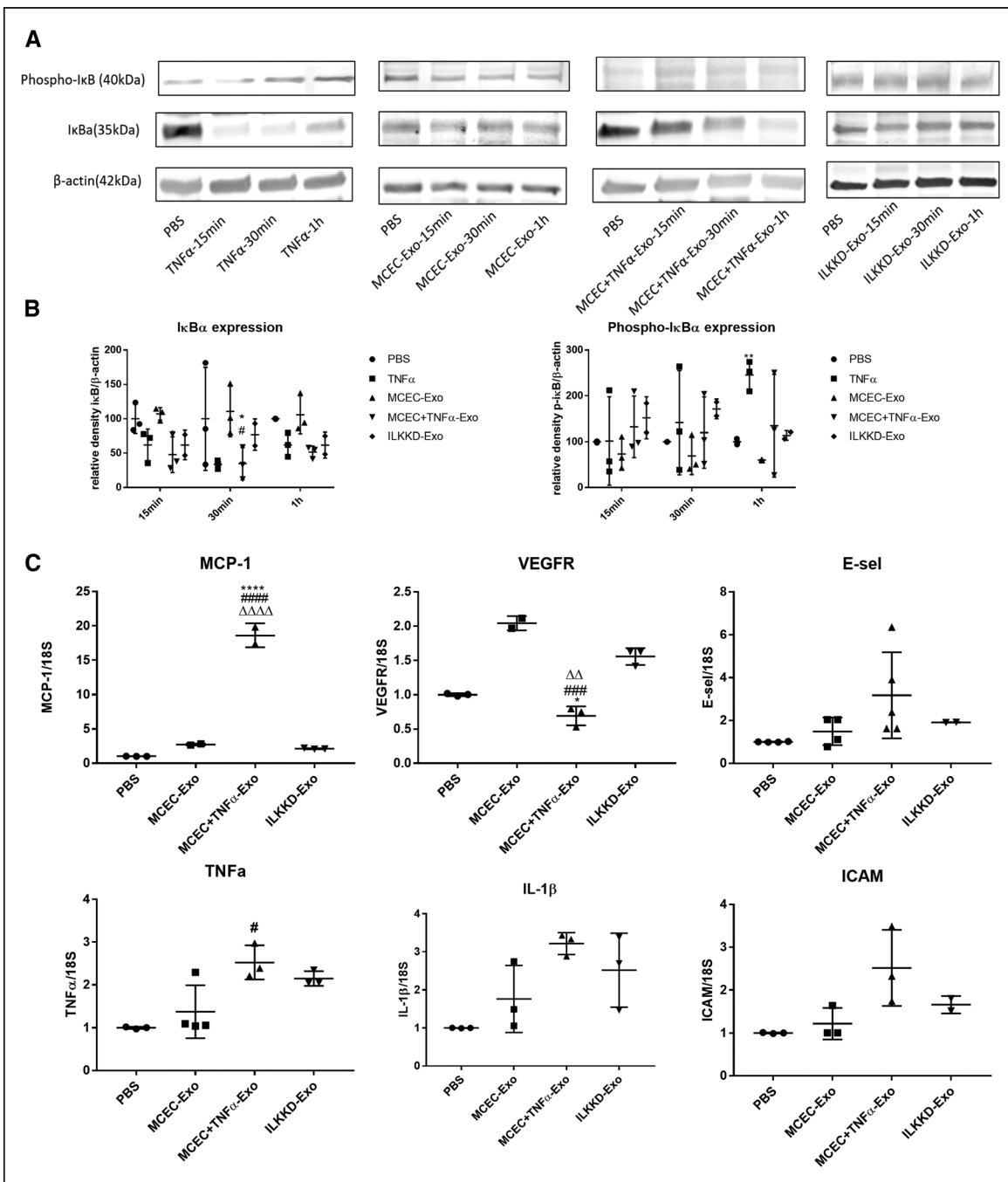


**Figure 5. ILK (integrin-linked kinase) knockdown exosomes inhibit NF-κB (nuclear factor κB) nuclear translocation.**

**A** and **B**, NF-κB staining analysis indicated nuclear NF-κB translocation was not activated with TNFα (tumor necrosis factor α) inflamed mouse cardiac endothelial cell (MCEC) with ILK knockdown-derived exosome (ILKKD-Exo) short-time treatment but activated with long time treatment (N=3, \*\*P=0.0087, \*\*\*\*P<0.0001; ##P=0.0015, ###P=0.0005, ####P<0.0001; Δ P=0.0121, ΔΔ P=0.0016, ΔΔΔ P<0.0001, ΔΔΔΔ P<0.0001; \*vs PBS group #vs MCEC-derived exosome [MCEC-Exo] group Δ vs ILKKD-Exo group; scale bar:10 μm). MCEC+TNFα-Exo indicates TNFα inflamed MCEC-derived exosome. DAPI indicates 4'6-diamidino-2-phenylindole; and HPF, high power field.

condition of parental cells.<sup>15</sup> Cardiovascular diseases are usually accompanied by ischemia, hypoxia, intense inflammatory stress, diabetes mellitus, or aging, which may dramatically

compromise stem cell cardiac repair ability<sup>35,48,49</sup> and induce dysfunctional exosome secretion.<sup>18,49</sup> Previous in vitro study from our lab revealed that IL-10 deficiency induced EPCs

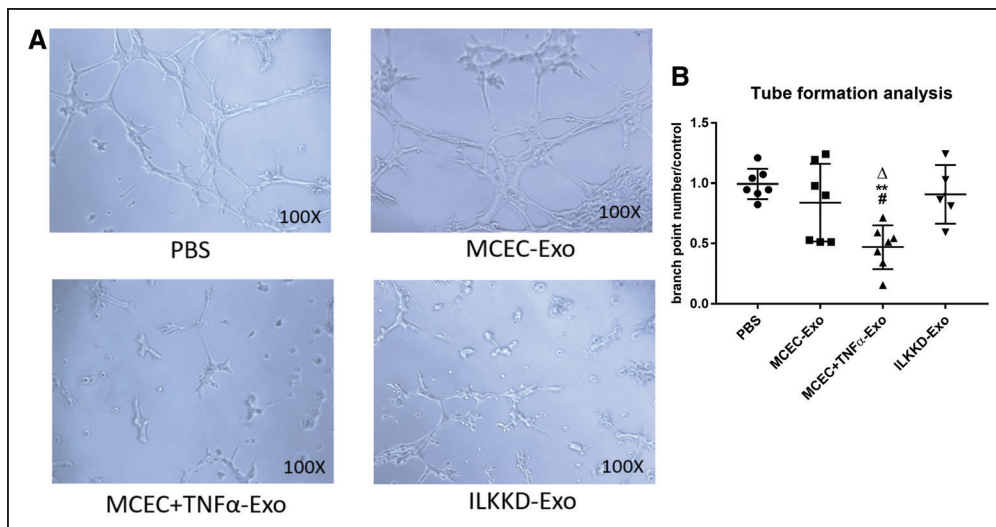


**Figure 6. ILK (integrin-linked kinase) knockdown exosomes attenuate NF-κB (nuclear factor κB) signaling pathway activation.** **A** and **B**, Western blotting showed short-time TNFα (tumor necrosis factor α) inflamed mouse cardiac endothelial cell with ILK knockdown-derived exosome (ILKKD-Exo) treatment inhibited IκBα (inhibitor of κBα) degradation (N=3, \*P=0.212, \*\*P=0.0076, #P=0.0235). **C**, Quantitative real-time polymerase chain reaction analysis indicated 24 h of ILKKD-Exo treatment showed lower level of NF-κB downstream gene expression (N=3, \*P=0.0371, \*\*P=0.0016, \*\*\*\*P<0.0001; ###P=0.0067, #####P<0.0001; ΔΔΔ P<0.0001; \*vs PBS group, #vs MCEC-derived exosome [MCEC-Exo] group, Δ vs TNFα inflamed MCEC with ILK knockdown-derived exosome [ILKKD-Exo] group). IL indicates interleukin; MCEC+TNFα-Exo, TNFα inflamed MCEC-derived exosome. E-sel indicates E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; MCEC+TNFα-Exo, TNFα inflamed MCEC-derived exosome; MCP, monocyte chemoattractant protein; pIκBα, phosphorylated IκBα; and VEGFR, vascular endothelial growth factor receptor.

exosome dysfunction in preserving cell survival, promoting cell mobilization and angiogenesis<sup>18</sup> and altered exosomal RNA and protein content.

To further enhance therapeutic effect of stem cell-derived exosomes, multiple studies have focused on

microRNA (miR) manipulation in exosomes. Our previous studies found overexpressing miR-294 in mouse embryonic stem cell exosomes increased survival, cell cycle progression, and proliferation of cardiac progenitor cells for MI treatment.<sup>34</sup> Knocking down miR-375 in



**Figure 7. ILK (integrin-linked kinase) knockdown in TNF $\alpha$  (tumor necrosis factor  $\alpha$ ) inflamed mouse cardiac endothelial cell-derived exosome (MCEC+TNF $\alpha$ -Exo) rescued angiogenesis dysfunction.**

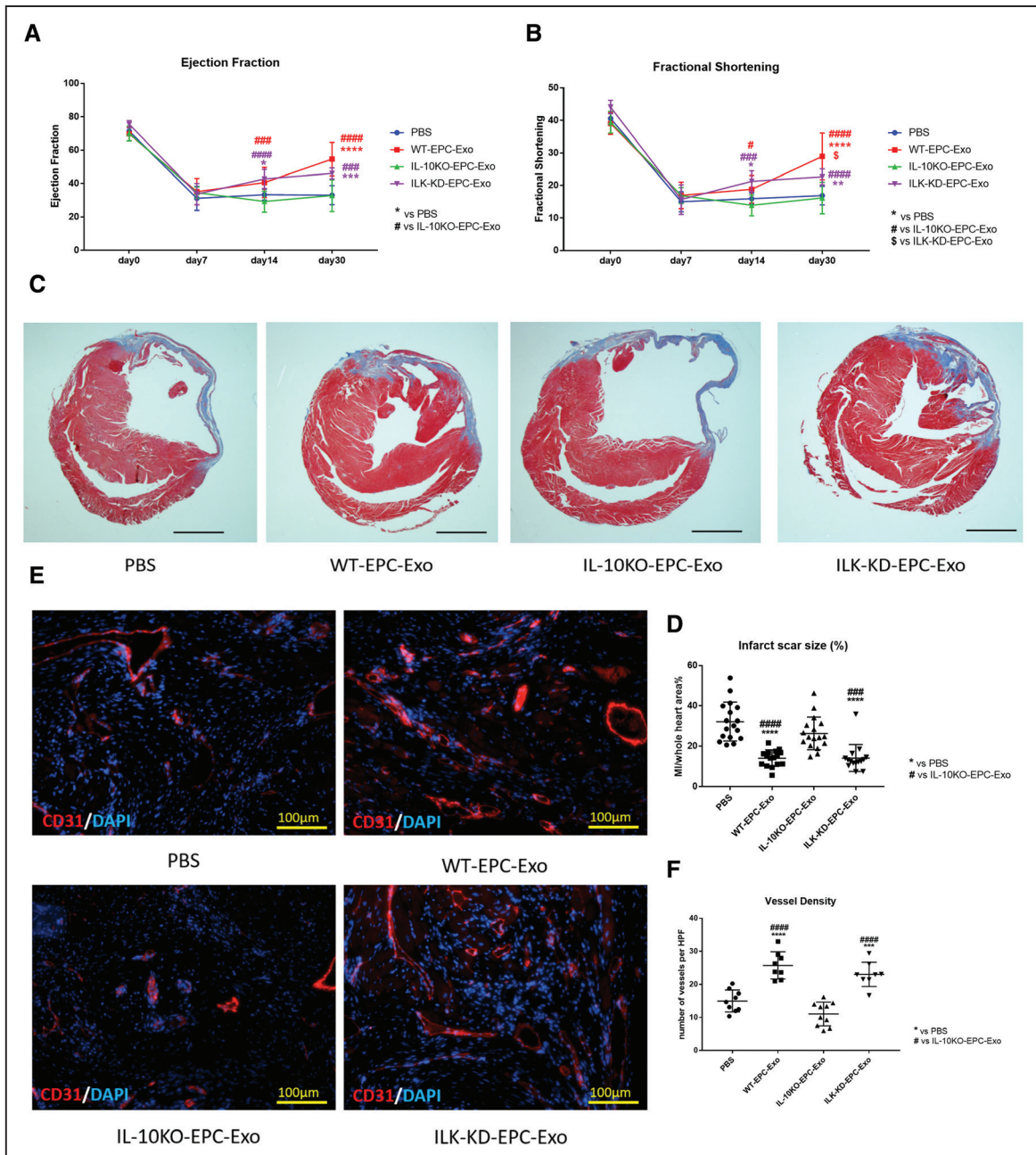
**A** and **B**, MCEC+TNF $\alpha$ -Exo inhibited tube formation while ILK knockdown in exosomes attenuated the inhibiting effect (N=5, \*\*P=0.016, #P=0.0304,  $\Delta$ P=0.0172; \*vs PBS group; #vs MCEC-derived exosome [MCEC-Exo] group;  $\Delta$ vs TNF $\alpha$  inflamed MCEC with ILK knockdown-derived exosome [ILKKD-Exo] group).

IL-10KO-EPC-Exo enhanced endothelial cells' ability against hypoxia stress.<sup>18,50</sup> Despite excellent progress made in miRs, very few studies have invested efforts to investigate the functional roles for exosomal protein cargo likely due to difficulties in stem cell culture and exosome protein collection. Our data on inflammation-induced augmentation of ILK in exosomes and a functional role of exosomal ILK in altering signaling and biology of recipient cells, therefore, opens new understanding of exosome biology and function. To rule out that observed ILK effect in IL-10KO mice is not due to loss of IL-10 itself, both BM-EPC (bone marrow-derived EPC)-Exo-treated and TNF-treated MCEC-derived exosomes were used for mechanistic experiments. We considered MCEC-Exo as a reasonable alternative strategy for several reasons, in context to protein content, both IL-10KO-EPC-Exo and MCEC+TNF $\alpha$ -Exo were enriched with ILK; functionally, we observed similar angiogenesis inhibiting effect in MCEC+TNF $\alpha$ -Exo. With ILK knockdown in exosomes, both IL-10KO-EPC-Exo and MCEC+TNF $\alpha$ -Exo showed increased angiogenic capacity. We think that the mechanistic studies conducted with MCEC-Exo should have a relatively good reflection of IL-10KO-EPC-Exo.

The role of ILK in heart and stem cells have been investigated for years but not fully understood largely due to its cell and context-specific roles. Although a study suggested that ILK-engineered mesenchymal stem cells showed enhanced cardiac therapeutic effect<sup>51</sup> other studies indicated ILK played negative role in cardiomyocytes. ILK was reported to be highly expressed in human failing heart and in cardiomyopathy.<sup>28</sup> In myocardial hypertrophy, ILK is central mediator of oxidative stress, inflammatory cell recruitment, and cardiac remodeling<sup>30</sup> and promotes profibrotic process and play critical role in

cardiac fibrosis under angiotensin II stimulation.<sup>52</sup> Moderate downregulation of ILK in heart prevented the decline in cardiac performance with aging.<sup>31,52</sup> The exact role of ILK is largely dependent on cell type and pathological condition. Specific to our study, we investigated the role of ILK-enriched exosomes in NF- $\kappa$ B activation and inflammatory response. Upon inflammatory stimulus (such as lipopolysaccharide, TNF $\alpha$ , etc), ILK can be activated through PI3K pathway and phosphorylates p65 subunit of NF- $\kappa$ B leading to the activation of NF- $\kappa$ B pathway. It has also been shown that ILK is not required for the inflammatory stimuli-induced classical NF- $\kappa$ B signaling pathway involving I $\kappa$ B $\alpha$  phosphorylation and degradation and nuclear translocation of NF- $\kappa$ Bp65. Rather, ILK utilizes alternate mechanisms for NF- $\kappa$ B activation and transcription activity through direct p65 phosphorylation at Ser536 and is required for lipopolysaccharide-induced transactivation of p65 through Ser536<sup>23</sup>. Our studies support that ILK contained in exosomes from inflamed cells utilizes the latter alternate mechanism of direct serine536 phosphorylation and nuclear translocation of NF- $\kappa$ Bp65.

NF- $\kappa$ B superfamily is known to play an essential role in inflammatory response and cardiac cell biology.<sup>19,53</sup> Some studies suggest NF- $\kappa$ B is cardioprotective during acute hypoxia and reperfusion injury.<sup>25,54</sup> However, prolonged activation of NF- $\kappa$ B appears to be detrimental and promotes heart failure by eliciting signals that trigger chronic inflammation through enhanced elaboration of cytokines, including TNF $\alpha$ , IL-1, and IL-6, leading to endoplasmic reticulum stress responses and cell death.<sup>25,55</sup> In our study, TNF $\alpha$  treatment of ILK knockdown MCEC-derived exosomes showed attenuated NF- $\kappa$ B activation in recipient endothelial cells. Knockdown of ILK in IL-10KO-EPCs rescued the functionality



**Figure 8. ILK (integrin-linked kinase) knockdown in IL-10 knockout endothelial progenitor cell–derived exosome (IL-10KO-EPC-Exo) rescued cardiac repair and regeneration dysfunction.**

**A** and **B**, Echocardiographic analysis followed for a month, ejection fraction and fractional shortening were used for left ventricle cardiac function measurement (N=10, in **A**, \* $P=0.0222$ , \*\*\* $P=0.0002$ , \*\*\*\* $P<0.0001$ , ### $P=0.0002$ , #### $P=0.0007$ , ##### $P<0.0001$ , in **B**, \* $P=0.0231$ , \*\* $P=0.0065$ , # $P=0.0163$ , ### $P=0.0001$ , #### $P<0.0001$ , \$ $P=0.0103$ ). **C** and **D**, Masson trichrome staining analysis on day 30 post-myocardial infarction (MI) heart for scar size measurement (scale bar: 2000  $\mu\text{m}$ , N=15, ### $P=0.0001$ , #### $P<0.0001$ , \*\*\*\* $P<0.0001$ ). **E** and **F**, CD31 staining on day 30 post-MI heart for angiogenesis measurement (scale bar: 100  $\mu\text{m}$ , N=9, \*\*\* $P=0.0005$ , \*\*\*\* $P<0.0001$ , ##### $P<0.0001$ ; \*vs PBS group; #vs IL-10KO-EPC-Exo group; \$vs IL-10 knockout EPC with ILK knockdown-derived exosome [ILK-KD-EPC-Exo] group). DAPI, 4'6-diamidino-2-phenylindole; and WT-EPC-Exo, wild-type EPC-derived exosome.

of their exosomes both in vitro and in vivo. Additionally, we found ILK knockdown also decreased expression of miR-375 (Online Figure VII), which has been shown to be detrimental for EPC survival, migration, angiogenesis, as well as myocardial repair. Our results indicated that ILK-enriched exosomes contributed to enhanced

inflammatory response through NF- $\kappa\text{B}$  activation, ILK enrichment is at least partially responsible for compromised cardiac repair and angiogenic ability of EPCs and EPC exosomes under inflammatory stimulus, and ILK manipulation significantly rescued or enhanced IL-10 deficiency induced EPC exosome dysfunction.

In summary, our study demonstrated that IL-10 deficiency, which mimics post-MI systemic inflammation condition, altered EPC-derived exosome content and function in myocardial repair largely via enhanced ILK packaging and ILK-mediated NF- $\kappa$ B-dependent augmentation in inflammatory gene expression. Our study identified ILK as a potential target for ischemic heart disease, broadened understanding of exosome-mediated inflammatory response, and raised possibility of exosome protein manipulation for cell-free therapeutic strategy for ischemic heart repair.

## ARTICLE INFORMATION

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### Disclosures

None.

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