Myocardial injury after ischemia-reperfusion in mice deficient in Akt2 is associated with increased cardiac macrophage density

Xue Li,1 Deana Mikhalkova,1 Erhe Gao,1 Jin Zhang,1 Valerie Myers,1 Carmen Zincarelli,1 Yonghong Lei,1 Jianliang Song,1 Walter J. Koch,1 Karsten Peppel,1 Joseph Y. Cheung,1 Arthur M. Feldman,1 and Tung O. Chan1,2

1Center for Translational Medicine, Department of Medicine, and 2Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania

Submitted 2 August 2010; accepted in final form 1 September 2011


Li X, Mikhalkova D, Gao E, Zhang J, Myers V, Zincarelli C, Lei Y, Song J, Koch WJ, Peppel K, Cheung JY, Feldman AM, Chan TO. Myocardial injury after ischemia-reperfusion in mice deficient in Akt2 is associated with increased cardiac macrophage density. Am J Physiol Heart Circ Physiol 301: H1932–H1940, 2011. First published September 2, 2011; doi:10.1152/ajpheart.00755.2010.—Akt2 protein kinase has been shown to promote cell migration and actin polymerization in several cell types, including macrophages. Because migrating macrophages constitute an important inflammatory response after myocardial ischemia, we determined cardiac macrophage expression after ischemia-reperfusion (I/R) injury and cryo-injury in mice lacking Akt2 (Akt2-KO). At 7 days post-I/R, Akt2-KO cardiac tissues showed an increase in immunohistochemical staining for macrophage markers (Galectin 3 and F4/80) compared with wild-type (WT) mice, indicating macrophage density was increased in the injured Akt2-KO myocardium. This change was time dependent because macrophage density was similar between WT and Akt2-KO myocardium at 3 days post-I/R, but by 7 and 14 days post-I/R, macrophage density was significantly increased in Akt2-KO myocardium. Concomitantly, infarct size was larger and cardiac function was reduced in Akt2-KO mouse subjects to I/R. However, when cryo-infarction produced similar infarct sizes in the anterior wall in both WT and Akt2-KO mice, macrophage density remained higher in Akt2-KO mouse myocardium, suggesting Akt2 regulates myocardial macrophage density independent of infarct size. Consistently, bone marrow from Akt2-KO mice enhanced myocardial macrophage density in both C57/B6 WT and Akt2-KO recipient mice. Finally, reciprocal ex-vivo coculturing of macrophages and cardiac myocytes showed that activated Akt2-KO peritoneal macrophages had reduced mobility and adhesion when compared with WT littermate controls. Thus, although Akt-2 KO mice did not affect the initial inflammation response after injury and Akt2 deficiency has been shown to impair cell migration or motility in macrophages, our data suggested a novel mechanism to explain increased cardiac macrophage density post-I/R injury and cryo-injury in Akt2-KO mouse subjects to I/R.

MATERIALS AND METHODS

I/R surgery in mice. Akt2-KO mice in C57/B6 background (6) were backcrossed for four additional generations with C57/B6. Homozygous Akt2-KO mice and congenic C57/B6 wild-type (WT) mice were used for I/R surgery as described previously (2, 16, 38). Briefly, age-matched male mice (8–12 wk, 22–24 g) were anesthetized with 2% isoflurane, and the heart was exposed through a left thoracotomy at the 5th intercostal space. The slipknot was tied around the left anterior descending (LAD) coronary artery 2 to 3 mm from its origin, and the heart was immediately returned to the chest cavity followed by evacuation of pneumothorax and closure of muscle and skin layers. The slipknot was released after 30 min of ischemia to allow reperfusion. Sham-operated animals were subjected to the same surgical procedures except that the slipknot was not tied. Animals recovered from anesthesia within 5 min after the completion of surgery and received ibuprofen (10 mg/50 ml drinking water) for 48 h as post-surgery analgesia. Under our experimental conditions, 76% WT and 74% Akt2-KO mice survived the first 5 days of surgery (P = not significant). Experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Thomas Jefferson University Committee on Animal Care.

Cryo-injury. The procedure was performed as described with modifications (35). Briefly, after anesthetized with 2.5% Avertin (10 μl/g body wt ip), the heart was exposed at the left 4th intercostal space. A 3 mm cryoprobe (liquid nitrogen cooled) was applied three times (10
s each) to the anterior left ventricular (LV) free wall. After each application, the probe was rinsed with saline at RT to allow nontraumatic probe detachment. Application of cryoprobe was guided using the left atrium and pulmonary artery as anatomic landmarks.

**Hemodynamic measurement.** We evaluated cardiac hemodynamics at 4 wk after I/R injury as described (11, 26). Briefly, a 1.4 F micromanometer catheter (Millar Instruments) was inserted into the LV through the right carotid artery of anesthetized mice. In vivo hemodynamics were recorded at the baseline and after administration of 10 ng of isoproterenol.

**Echocardiography.** Echocardiographic studies were performed using an ultrasonicographic system (ACUSON Sequoia C256) as described (14). Briefly, a 14-MHz transducer was applied to the left hemithorax of mice anesthetized with 2.5% Avertin (10 μl/g body wt ip). M-mode imaging was obtained from the short-axis view at the level of the greatest LV dimension at baseline.

**Determination of myocardial apoptosis.** Myocardial apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining and by caspase-3 activity as we described recently (16, 17). Briefly, for TUNEL staining, hearts were visualized under a fluorescence microscope with the DAPI filter, 6-diamidino-2-phenylindole (DAPI). The entire population described recently (16, 17). Briefly, for TUNEL staining, hearts were nick-end labeling (TUNEL) staining and by caspase-3 activity as we analyzed by terminal deoxynucleotidyl transferase dUTP-mediated reaction (TUNEL). M-mode imaging was obtained from the short-axis view at the level of the greatest LV dimension at baseline.

**Infarct size measurement.** Myocardium was stained with 2% triphenyltetrazolium chloride (TTC) to measure infarct size as described (16). Briefly, 72 h after I/R, the slipknot around LAD was retied. After the injection of 2% Evans Blue dye (0.2 ml), mouse heart was quickly excised, and LV was sliced into five equally thick sections perpendicular to the short axis of the heart and incubated in PBS containing TTC. After 15 min at RT, the slices were digitally photographed. The Evans Blue-stained area (area not at risk), TTC-negative area (infarcted myocardium) and area at risk (include both TTC-negative and positive areas) were measured with the computer-based image analyzer SigmaScan Pro 5.0 (SPSS Science, Chicago, IL). Myocardial infarct size and AAR were expressed as percentage of total LV.

For measurement of infarct size at 7 days and 14 days after surgery, hearts were excised and fixed with 4% paraformaldehyde for 24 h. LV slices were paraffin-embedded, stained with hematoxylin-eosin and Masson’s Trichrome C, and digitally photographed. Infarct and collagen areas were determined as above. Power fields (20x) from 7 to 8 mice/group were analyzed to determine the percent infarction per LV area.

**Histology and immunohistochemistry.** Histology and immunohistochemistry samples were routinely processed by the Research Animal Diagnostic Laboratory (University of Missouri). For histology, 5 μm sections of fixed tissue were stained with hematoxylin-eosin and Masson’s Trichrome C and digitally photographed. For immunostaining, markers for macrophages (F4/80, Galectin 3) and neutrophils (Gr-1) were performed in heart samples fixed in IHC-Zinc Fixative (BD Pharmingen) for 8 h and transferred into 70% ethanol for storage.

Paraffin-embedded tissue sections (5 μm) were subjected to heat-induced epitope retrieval in a 10 mM citrate buffer (pH 6.0) for 20 min with a 20 min cool down. All tissues were incubated with dilute H2O2 (0.03%) and peroxidase-labeled streptavidin (DAKO) for 30 min. Bound antibodies were visualized following incubation with 3,3′-diaminobenzidine solution (0.05% with 0.015% H2O2 in PBS; DAKO) for 3–5 min. Mouse spleen sections were probed for positive control, and sections of experimental tissue were incubated with isotype-matched normal antibody as negative control. Sections were counterstained with Meyer’s hematoxylin and dehydrated for microscopic examination. For quantification, infarct areas were imaged using identical exposure times and incident light intensities. Images were digitally quantified using ImageJ system (National Institutes of Health 1.42).

**Real-time quantitative PCR.** Real time PCR was performed in 50 μl reaction (5 μl cDNA; 250 nM each primer; 1X SYBR Green Master Mix). Three samples were measured in each experimental group in triplicate in a minimum of two independent experiments. ΔCT method was used for this study, and the results were presented as relative fold changes of GAPDH gene. Mouse gene specific primers sets were as follows: Galectin 3 (5′ ATG ACC TGC CCT TGC CTG 3′, 5′ CTT GCT TCG TGT TAC ACA CAA TG3′); monocyte chemoattractant protein (MCP-1; 5′AGG TTC CTG TCA TGC TG3′, 5′GCT GCC TTC TTC TCG CA′); Gr-1 (5′ CTG CAA CAA GCC CAG GGA GGA C3′, 5′ TCG TGC AAC GAG GCA GCC CGC C′); IL-10 (5′ TGC TTC CTG CAC CCC AAG GGA CGG GGG 3′, 5′TCT GGC ATC AGC TTC AGG CGC C3′); monocyte inducible nitric oxide synthase (iNOS; 5′ GCC AGG TGG TGC TGA GCT G 3′, 5′ GCT GCC TTC TTC TCG TCA′); Arginase (5′ CTG CAA CAA GCC CC 3′, 5′ATC TTC CAG AAC AGC GCC ACC′); TNF (5′GCA GGA GCT TCC GAC GGC AGG 3′, 5′ GCT GCT GCC CCT GCC CCC AAG 3′, 5′GCC CAG AGG TGA GGC AGC CA′); GAPDH (5′ AAC GAC CCC TTC ATT GAC 3′, 5′TCC AGC ACA TAC TCA GAC′).

Each primer set was designed assuming a melting temperature of 60°C and 50% GC content.

**Bone marrow transplantation.** We performed bone marrow transplantation in C57/b6 mice and congenic Akt2-KO mice using our previously established method (41). Briefly, 15-wk-old C57/b6 WT and congenic Akt2-KO mice received 950 rads of γ-irradiation and were administered with the antibiotic, Bayril. The next day, fresh bone marrow cells were isolated from a separate cohort of nonirradiated C57/b6 WT or congenic Akt2-KO mice (2 mice/group and pooled). Bone marrow cells (6 × 10⁶) in 200 μl volume were injected into irradiated mice through the ocular. Fourteen mice received bone marrow transplant, and one mouse died 2 wk after bone marrow transplantation. Five weeks after bone marrow transplantation, mice were used for I/R surgery. Under our experimental conditions, all mice survived the surgery. Seven days after surgery, mouse hearts were harvested for immunohistochemistry staining.

**Coculturing of thioglycollate-activated peritoneal macrophages with H9C2 cells and cardiac myocytes.** Briefly, littermates of WT and AKT-KO mice (derived from Akt2-KO heterozygous mating) were injected with 0.5 ml of 3% thioglycollate medium (BD BBL Thiglycollate Medium, Brewer Modified, Cat. No. 211716). Thioglycollate-activated peritoneal macrophages were isolated and labeled with PKH26-red fluorescent nontoxic membrane dye (Sigma; excitation 551 nm and emission 567 nm). On the same day, a different cohort of littermate WT and Akt2-KO mice were used to harvest adult cardiac myocytes as we described previously (3, 19). About 5,000 myocytes/glass slide were cultured in 24-well plates. Macrophages were suspended in myocyte growth medium and added to myocytes (100,000 macrophages/well in 0.5 ml medium) and cultured for 6 or 24 h as indicated. Alternatively, H9C2 cells derived from rat neonatal hearts (ATCC) were cultured in M199 medium supplemented with 10% fetal calf serum and antibiotics.
Statistical analysis. A commercial software package was used for all statistical analysis (Graph Pad Software; La Jolla, CA). Nonparametric one-way ANOVA (Kruskal-Wallis test with Dunn post-test) was used to analyze macrophage clustering area size. Mann-Whitney nonparametric methods were used to compare between two groups. A $P$ value of $<0.05$ was considered statistically significant.

RESULTS

Before I/R surgery, there were no differences between WT and Akt2-KO adult mice with respect to cardiac function (Table 1). In addition, these mouse hearts did not have detectable mRNA for the macrophage-marker Gal3 (Fig. 1A). Seven days after I/R surgery, mRNA levels for Gal3 were significantly increased in the infarct region of Akt2-KO myocardium ($3.7 \pm 1.2$ vs. WT-I/R; Fig. 1A). Immunohistochemistry staining corroborated that Gal3 expression density was significantly higher in the infarct Akt2-KO myocardium (Fig. 1B). We obtained similar results when we stained for another macrophage marker, F4/80 (Fig. 1C).

Table 1. Echocardiography of WT and Akt2-KO mice at 7 days after I/R surgery

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>WT I/R</th>
<th>Akt2-KO I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Percent fractional shortening</td>
<td>48.2 ± 2.9</td>
<td>47.4 ± 1.2</td>
<td>25.9 ± 0.4</td>
</tr>
<tr>
<td>End diastolic dimension, mm</td>
<td>2.88 ± 0.03</td>
<td>2.60 ± 0.12</td>
<td>3.67 ± 0.15</td>
</tr>
<tr>
<td>End systolic dimension</td>
<td>1.68 ± 0.10</td>
<td>1.60 ± 0.08</td>
<td>2.72 ± 0.14</td>
</tr>
<tr>
<td>Heart rate</td>
<td>450 ± 11</td>
<td>438 ± 11</td>
<td>411 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SE. Echocardiography of male wild-type (WT) and mice lacking Akt2 (Akt2-KO) mice at 7 days after ischemia-reperfusion (I/R) surgery. $P < 0.01$ vs. WT-I/R.

Three days post-I/R, macrophage density was similar between WT and Akt2-KO mice, suggesting myocardial monocyte recruitment and its differentiation into macrophages were similar between WT and Akt2-KO mice (Fig. 2A). Cardiac density of neutrophils at 1 day after I/R was also similar.

AJP-Heart Circ Physiol • VOL 301 • NOVEMBER 2011 • www.ajpheart.org
between WT and Akt2-KO mice (data not shown). However, by 7 and 14 days post-I/R, macrophage density was significantly higher in Akt2-KO myocardium compared with WT mice (Fig. 2A). The macrophage density correlated with cardiac function. At 1 day after I/R, fractional shortening in both WT and Akt2-KO mice was similarly depressed compared with sham-operated animals. Subsequently, cardiac function in Akt2-KO mice recovered more slowly compared with control mice (Fig. 2B and Table 1). At 28 days after I/R surgery, Akt2-KO mice had significantly reduced hemodynamic response and developed larger ventricular scar, LV dilatation, and hypertrophy compared with WT mice (Table 2 and data not shown).

DeBosch et al. (9) have shown that at 7 days after myocardial infarction, Akt2-KO mice had increased TUNEL-positive apoptotic cells when compared with WT-type infarcted mice. We found that even at 3 h after I/R injury, both TUNEL index (Fig. 2C) and caspase-3 activity (Fig. 2D) were significantly increased in Akt2-KO myocardium, suggesting Akt2-KO mice had increased apoptotic cells when compared with WT-type infarcted mice. Consistently, Akt2-KO mice sustained larger infarcts at 3 days post-I/R (Fig. 2E). Although at 7 days after I/R injury, the infarct size in Akt2-KO mice remained bigger compared with control mice, viable myocytes isolated from WT and Akt2-KO left ventricles did not show significant differences in myocyte size and calcium transients response (data not shown). However, at 28 days after I/R injury, Akt2-KO mice had worst global LV dilation and hypertrophy (Table 2) and increased in cardiac myocyte diameter in histologic cross-sections (data not shown).

To determine whether differences in infarct sizes between WT and Akt2-KO mice subjected to I/R were responsible for elevated macrophage density, we produced equivalent cryo-injury in both WT and Akt2-KO mice by using a liquid nitrogen-cooled 3 mm cryoprobe (Fig. 3A). At 1 wk after cryo-injury, macrophage density was higher in Akt2-KO compared with WT myocardium (Fig. 3B). Consistently, quantitative RT-PCR of mRNA isolated from cryo-infarcted myocardium showed that macrophage function markers, Galectin 3, MCP-1, IL-10, and iNOS, as well as arginase and TNF-α were all increased in the myocardium of cryo-infarcted Akt2-KO mice (data not shown), suggesting cardiac injury caused by I/R and cryo-infarction increased macrophage density in Akt2-KO hearts. When examined at 4 wk after cryo-injury, fractional shortening in Akt2-KO hearts was significantly reduced (Fig. 3C), suggesting enhanced macro-
phage density in Akt2-KO hearts contributed to depressed cardiac function. Ly6C is known to mark early inflammatory phase (28). We did not find a significant difference in Ly6C staining between WT and Akt2-KO myocardium at 4 days, 7 days, and 14 days after I/R (data not shown). It should be noted that Ly6C staining in myocardium were sparse compared with mouse spleen. To further characterize cardiac macrophage subsets, we determined the expression of iNOS, a marker inflammatory (M1) macrophages and IL-10, a marker for anti-inflammatory (M2) macrophages. Both iNOS and IL-10 mRNA were both significantly increased in Akt2-KO myocardium at 7 days after I/R compared with WT mice (Fig. 4, A and B), suggesting Akt2-KO affected changes in both macrophage subtypes.

MCP-1 is early induced after ischemia injury and is associated with macrophage infiltration by stimulating monocyte activation as well as production of transforming growth factor-β and collagen by fibroblasts (12, 13). Indeed, we found over 100-fold MCP-1 mRNA induction at 24 h after I/R in both WT and Akt2-KO myocardium compared with sham-operated hearts (data not shown). By 7 days post-surgery, MCP-1 mRNA elevation were reduced to only about 10-fold above sham-operated hearts (data not shown).

Table 2. Organ weights and hemodynamics at 28 days after I/R surgery

<table>
<thead>
<tr>
<th>Organ ratio</th>
<th>WT</th>
<th>Akt2-KO</th>
<th>I/R</th>
<th>Akt2-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Ventricular weight/body weight, mg/g</td>
<td>3.44 ± 1.11</td>
<td>3.90 ± 0.16</td>
<td>4.70 ± 1.17</td>
<td>6.19 ± 0.65*</td>
</tr>
<tr>
<td>Heart rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Beats/min</td>
<td>450 ± 11</td>
<td>438 ± 11</td>
<td>434 ± 29</td>
<td>410 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SE. Baseline and isoproterenol (5 ng) stimulated cardiac hemodynamic functions were recorded 28 days after I/R surgery. +dP/dt and −dP/dt: maximal 1st time derivatives of left ventricular pressure rise and fall, respectively. *P < 0.05 vs. WT-I/R.

Fig. 3. WT and Akt2-KO male mice (8 to 12 wk old) were subjected to cryo-injury and analyzed at 7 days after surgery. A: cryo-infarcted WT and Akt2-KO mouse hearts in 5 equal cross sections were stained with Trichrome C and imaged at 20× power fields. Infarcted areas were selected and quantified using computer-based image analysis software. Seven to eight mice per group were analyzed to determine the percent infarct/LV area. Signals were normalized to WT hearts. *P < 0.05 vs. WT-Cryo. B: myocardial immunostaining of F4/80 after cryo-injury. Infarcted area were imaged at 200× and digitally quantified as mean staining density. At least 5 hearts and 5 images from infarcted region of each heart were quantified. *P < 0.05 vs. WT-cryo. C: FS% 4 wk after cryo injury. Mice (6 to 7) were used in each time point. *P < 0.05 vs. WT. *P < 0.05 vs. WT-cryo. Mann-Whitney test was used for all analyses. Means are ± SE.
operated hearts (Fig. 4C). However, Akt2-KO myocardium had significantly higher levels of residue MCP-1 mRNA compared with WT controls (~3-fold increase vs. WT-I/R; Fig. 4C). In addition, despite similar cryo-infarct sizes (Fig. 3A), MCP-1 mRNA levels were significantly elevated in Akt2-KO cryo-infarcts (~3-fold vs. WT-cryo-infarct; Fig. 4D). These data suggest that genetic absence of Akt2 is associated with excess MCP-1 production after myocardial injury.

Small interfering RNA knockdown of Akt2 in mouse macrophages impair macrophage migration and associate with reduced the phosphorylation of serine 3 of cofilin, a site that regulates actin dynamics and cell migration (40). Similarly, we found that cofilin phosphorylation was significantly reduced in in vitro differentiated Akt2-KO mouse macrophages compared with WT macrophages (data not shown). To determine whether bone marrow or myocardium Akt2 deletion is augmenting macrophage density, we performed reciprocal bone marrow transplantation between C57/b6 mice and congenic Akt2-KO mice using our previously established method (41). After recovery from transplantation, mice were subjected to I/R surgery and mouse hearts were harvested after 7 days. Quantification of macrophage by F4/80 staining (Fig. 5, A and B) showed that bone marrow from Akt2-KO mice enhanced myocardial macrophage density in both C57/B6 WT and Akt2-KO recipient mice. We confirmed macrophage density changes with another macrophage marker, CD-68 (data not shown). Thus, Akt2-KO bone marrow enhanced macrophage density in the myocardium.

Finally, we developed an assay to coculture activated macrophages with cardiac myocytes ex vivo. To facilitate the monitoring of activated macrophages after mixing with cardiac myocytes, thioglycollate-activated peritoneal macrophages were prelabeled with a fluorescent nontoxic membrane dye, PKH26-red or PKH67-green. We noted that PKH26-red dye gave the best signal-to-noise images under fluorescence when cocultured with H9C2 heart cells or with cardiac myocytes.
(Fig. 6A and data not shown). We then cocultured macrophages and cardiac myocytes between Akt2-KO mice and their WT littermates. To this end, dispersed thioglycollate-activated peritoneal macrophages were suspended in myocyte growth medium and added to myocytes (100,000 macrophages to 5,000 laminin-adhered myocytes). We noted that after 6 h of coculture with cardiac myocytes, peritoneal-activated macrophages from WT mice migrated or adhered to each other, whereasAkt2-KO macrophages seeded at the same density remained immobile and failed to adhere (Fig. 6B and data not shown). The effect was maintained over 24 h (data not shown). Thus our novel coculture assay suggests mobility defects in Akt2-KO macrophages within the cardiac myocyte culture environment.

**DISCUSSION**

It is well established that the Akt kinase family plays important roles in protecting the heart from noxious stimuli and ameliorates LV hypertrophy and failure (reviewed in Ref. 32). For example, gene transfer of activated Akt1 protects mouse myocardium against acute injuries of I/R. Overexpression of an activated Akt1 mutant (Akt-E40K) or nuclear targeted Akt1 decreases apoptosis and fibrosis and improves cardiac function after trans-aortic constriction (TAC) surgery. Viewed in a broader context, we have shown previously that Akt phosphorylation is decreased in two models of dilated hypertrophy: mice that constitutively overexpressed either TNF-α or the adenosine A1 receptor (15, 20). However, mechanisms by which the Akt kinase family affords cardiac protection are complex since these kinases regulate a diverse array of functions within the cardiovascular system, including apoptosis, proliferation, differentiation, intermediary metabolism, and cell size (reviewed in Ref. 32). In addition, cardiac-expressed Akt (E40K mutation or nuclear-targeted Akt) can directly regulate cardiac contractility and Ca2+ homeostasis (7, 23, 29). Akt effects also are modulated by timing of Akt activation since short-term activation of Akt causes an enlarged heart without significant pathology (7, 8, 25, 30, 33), but chronic Akt overexpression results in dilated cardiomyopathy and loss of angiogenesis (31).

Adding to the complexity, the three highly homologous members of Akt kinase family (Akt1, Akt2, and Akt3) can differentially affect cardiac responses to physiological or pathological stimuli. For example, Akt1-KO mice are resistant to swimming training-induced cardiac hypertrophy, but develop an exacerbated cardiac hypertrophy 7 days after TAC surgery (9). By contrast, although cardiac responses post-TAC are similar between WT and Akt2-KO mice, permanently occluding LAD coronary artery results in larger infarcts in Akt2-KO mice at 7 days, despite similar infarct sizes measured at 24 h post-occlusion (9, 10). This is likely due to higher apoptosis rate in Akt2-KO animals (10). In addition to its well-established prosurvival and growth functions, Akt2 has also been shown to promote cell migration and actin polymerization in breast cancer cells through β1 integrin and vimentin (21) and in glioma cells and macrophages by promoting phosphorylation of girdin or coflin (24, 39, 40). In agreement with these reports, we showed that Akt2-KO mouse-derived macrophages had reduced coflin phosphorylation but normal expression of integrin β1, integrin β3, and vimentin.

To explore novel mechanisms by which Akt2 affords cardioprotection following insults, we used two fundamentally different models of myocardial injury: I/R and cryo-infarction. We demonstrated that genetic absence of Akt2 resulted in worsening cardiac contractility during recovery compared with WT mice. However, elevated macrophage density in injured Akt2-KO myocardium post-I/R was not due to larger infarct size. Despite similar infarct sizes induced by cryoprobe in both WT and Akt2-KO hearts, macrophage density was higher in Akt2-KO mouse myocardium. This observation suggests that Akt2 directly regulates myocardial macrophage function independent of infarct size. In addition, despite similar cryo-infarct sizes, contractility was worse in Akt2-KO hearts in which macrophage density was significantly higher, suggesting enhanced macrophage presence, by itself, had deleterious effects.
on cardiac function. We should note that it remained to be determined whether augmented macrophage density is directly responsible for the observed decline in cardiac function.

Having established cardiac injury models in which Akt2 plays important roles in repair and recovery, we tested the hypothesis that Akt2 mediates cardioprotection by its effect on macrophage migration/retention. At early stages of injury (<3 days post-I/R), neither neutrophil nor macrophage density was different between WT and Akt2-KO hearts, suggesting Akt2 had little to no effects on the initial recruitment of monocytes and macrophages to the injured myocardium. In addition, infarct sizes determined at 3 days post-I/R were already larger in Akt2-KO hearts (at a time when macrophage density was similar), suggesting that mechanisms other than the effect of Akt2 on macrophage recruitment/retention account for its salutary effects during the early phase of I/R injury. In the intermediary stages of cardiac repair and recovery, genetic absence of Akt2 resulted in higher macrophage density at 7 to 14 days post-I/R, a period during which the granulating tissues are maturing.

Our studies do not support the model by which the increased myocardial macrophage density in Akt2-KO myocardium is caused by greater infiltration of undifferentiated macrophages (monocyte) after initial cardiac damage. Instead, our studies suggest a novel mechanism in which the increased Akt2-KO macrophage density is caused by enhanced retention of Akt2-KO macrophages in the myocardial space.

This novel mechanism of macrophage retention is supported by our newly developed assay of reciprocal coculturing macrophages and cardiac myocytes (Fig. 6). Using this assay, we showed that after 6 h of coculturing, macrophages derived from Akt2-KO mice remained immobile and failed to adhere with each other compared with WT macrophages, suggesting a mobility defect in Akt2-KO macrophages. In support, Akt2 deficiency has been shown to impair cell migration or motility in many cell types, including macrophages (4, 5, 21, 39, 40). Consistently, although the density of Akt2-KO macrophages in the myocardium was increased at 7 days after surgery, this was not the case at early stages of injury. Neither neutrophil density (24 h, data not shown) nor macrophage density (3 days; Fig. 2A) and the early inflammatory marker, Ly6C (3 days, data not shown), were increased, suggesting Akt2 kinase deletion has little or no effect on the initial recruitment of neutrophils and monocytes into the injured myocardium.

Our bone marrow transplantation experiments provided in vivo support for this model. After transplantation and I/R surgery, the density of Akt2-KO macrophages was increased in both WT and Akt2-KO myocardium. Because Akt2 deletion did not affect the initial inflammation response and Akt2-KO macrophages have impaired motility response, the most likely explanation for the increased macrophage density is an increase in the retention of Akt2-KO macrophages in the myocardial space.

Higher macrophage density is correlated with elevated expression of macrophage functional markers such as class A scavenger macrophage receptor and transforming growth factor-β (data not shown). Class A scavenger macrophage receptor has been implicated in cellular and pathogenic clearance, and its expression is induced by the binding of lipopolysaccharides as well as dead or apoptotic cells (27). Elevated macrophage density in the injured myocardium is associated with delayed recovery in Akt2-KO mice, as indicated by depressed fractional shortening and in vivo hemodynamic performance 28 days post-I/R.

Our current studies suggest a novel mechanism in which Akt2 regulates cardiac macrophage density through macrophage retention. Several mechanisms could mediate this effect. First, Akt2 may regulate cardiac macrophage density via MCP-1. MCP-1 has been long recognized as a chemoattractant for monocyte/macrophages and lymphocytes (18, 22). We noted that although MCP-1 expression is normally most robust within 24 h after cardiac injury, we detected significantly more MCP-1 mRNA in Akt2-KO myocardium compared with WT controls at 7 days after cryo or I/R surgery. Thus excess MCP-1 after myocardial injury in Akt2-KO myocardium may account for increased macrophage density, inflammatory response, and interstitial fibrosis. Alternatively, macrophages interact with cardiac myocytes through intercellular adhesion molecule-1 (ICAM-1) (34), suggesting ICAM-1 interaction could contribute to the changes in density. Finally, macrophage Akt1 regulates microRNAs such as Let-7e, miR155, and miR125b and through which affects inflammation regulators such as SOCS1 and SHIP1, TNF-α, and the toll-like receptor 4 (1). Akt2 may differentially regulate the same or different sets of microRNAs to affect macrophage adhesion.

However, the precise deciphering of the roles of MCP-1, ICAM-1, and microRNAs in regulating Akt2-mediated macrophage retention in the myocardium will require tissue-specific and time-controlled Akt2 expression within the injured myocardium as well as the use of ex vivo coculturing of macrophages and cardiac myocytes. In summary, we propose a mechanism by which Akt2 protects cardiac injury by inhibiting excessive macrophage density in injured myocardium, in addition to its established prosurvival functions.

**GRANTS**

This work was supported by the National Heart, Lung, and Blood Institute Grant P01-HL-091799-01 (to A. M. Feldman and T. O. Chan), HL-58672, and HL-74854 (to J. Y. Cheung) and R37-HL061690 and P01-HL075443 (project 2, to W. J. Koch) and Pennsylvania Research Formulary Fund and American Heart Association Grant SDG-F64701 (to T. O. Chan).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


