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Therapeutic administration of IL-11 exhibits the postconditioning effects against ischemia-reperfusion injury via STAT3 in the heart

Masanori Obana,[1] Kaori Miyamoto,[1] Shiho Murasawa,[1] Tomohiko Iwakura,[1] Akiko Hayama,[1] Tomomi Yamashita,[1] Momoko Shiragaki,[1] Shohei Kumagai,[1] Akimitsu Miyawaki,[1] Kana Takewaki,[1] Goro Matsumiya,[2] Makiko Maeda,[3] Minoru Yoshiyama,[4] Hiroyuki Nakayama,[1] and Yasushi Fujio[1]

[1]*Laboratory of Clinical Science and Biomedicine, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan;* [2]*Department of Cardiovascular Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan;* [3]*Department of Clinical Pharmacogenomics, School of Pharmacy, Hyogo University of Health Sciences;* [4]*Department of Internal Medicine and Cardiology, Graduate School of Medicine, Osaka City University, Osaka, Japan*

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Obana M, Miyamoto K, Murasawa S, Iwakura T, Hayama A, Yamashita T, Shiragaki M, Kumagai S, Miyawaki A, Takewaki K, Matsumiya G, Maeda M, Yoshiyama M, Nakayama H, Fujio Y. Therapeutic administration of IL-11 exhibits the postconditioning effects against ischemia-reperfusion injury via STAT3 in the heart. *Am J Physiol Heart Circ Physiol* 303: H569–H577, 2012. First published June 15, 2012; doi:10.1152/ajpheart.00060.2012.—Activation of cardiac STAT3 by IL-6 cytokine family contributes to cardioprotection. Previously, we demonstrated that IL-11, an IL-6 cytokine family, has the therapeutic potential to prevent adverse cardiac remodeling after myocardial infarction; however, it remains to be elucidated whether IL-11 exhibits postconditioning effects. To address the possibility that IL-11 treatment improves clinical outcome of recanalization therapy against acute myocardial infarction, we examined its postconditioning effects on ischemia/reperfusion (I/R) injury. C57BL/6 mice were exposed to ischemia (30 min) and reperfusion (24 h), and IL-11 was intravenously administered at the start of reperfusion. I/R injury mediated the activation of STAT3, which was enhanced by IL-11 administration. IL-11 treatment reduced I/R injury, analyzed by triphenyl tetrazolium chloride staining [PBS, 46.7 ± 14.4%; IL-11 (20 μg/kg), 28.6 ± 7.5% in the ratio of infarct to risk area]. Moreover, echocardiographic and hemodynamic analyses clarified that IL-11 treatment preserved cardiac function after I/R. Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling staining revealed that IL-11 reduced the frequency of apoptotic cardiomyocytes after I/R. Interestingly, IL-11 reduced superoxide production assessed by in situ dihydroethidium fluorescence analysis, accompanied by the increased expression of metallothionein 1 and 2, reactive oxygen species (ROS) scavengers. Importantly, with the use of cardiac-specific STAT3 conditional knockout (STAT3 CKO) mice, it was revealed that cardiac-specific ablation of STAT3 abrogated IL-11-mediated attenuation of I/R injury. Finally, IL-11 failed to suppress the ROS production after I/R in STAT3 CKO mice. IL-11 administration exhibits the postconditioning effects through cardiac STAT3 activation, suggesting that IL-11 has the clinical therapeutic potential to prevent I/R injury in heart.

cardiovascular diseases; cytokine; signal transduction

ISCHEMIA-REPERFUSION (I/R) is one of the major causes of myocardial injury in the clinical setting, especially in the therapeutic process of acute myocardial infarction. Although various kinds of preventive therapies from I/R injury have been proposed so far, clinical trials revealed that they are insufficient. Therefore, it is urgent to develop the therapeutic strategy on a novel concept to prevent myocardial damage after I/R. Accumulating evidence has shown that cardiac homeostasis is maintained by a wide range of neurohumoral factors and cytokines, suggesting that these factors could be therapeutic targets for cardioprotection.

IL-6 family cytokines contribute to cardioprotection by activating various kinds of signaling molecules. In their cytokine signaling pathways, activation of glycoprotein 130/STAT3 axis plays important roles in cytoprotection and angiogenesis (6, 21, 22). Thus the activation of STAT3 by IL-6 family cytokines is considered a potential therapeutic strategy for cardiovascular diseases (5). Experimentally, leukemia inhibitory factor shows the antifibrotic effect after myocardial infarction (29); however, this cytokine has not been considered clinically appropriate because of its proinflammatory properties (7). To establish a novel therapeutic strategy against cardiovascular disease, we focused on IL-11, a member of IL-6 family cytokines, because its proinflammatory activity is limited and because IL-11 exhibits anti-inflammatory activity in some cases (3, 23).

IL-11 exhibits multipotential functions (4). Because IL-11 has the thrombopoietic activity, recombinant human IL-11 is clinically used for thrombocytopenia (9). In addition, IL-11 also shows nonhematopoietic functions. Previously, we reported that IL-11 protects cardiomyocytes from H_2O_2-induced cell death through STAT3 activation and has a late preconditioning effect against I/R injury (16). Recently, we also demonstrated that the therapeutic treatment of IL-11 reduces adverse cardiac remodeling after myocardial infarction in murine model, concomitant with anti-apoptosis and angiogenesis (20). Furthermore, cardiac-specific ablation of STAT3 abrogated IL-11-mediated attenuation of adverse cardiac remodeling, suggesting that cardiac activation of STAT3 mediates antifibrotic effects.

In this study, to address the possibility of clinical application of IL-11 treatment in the therapeutic process of acute myocardial infarction as a cardioprotective strategy, we investigated its postconditioning effects on I/R injury. In addition, we examined whether IL-11 utilizes the cardiac STAT3 signaling pathway in its postconditioning effects.

MATERIALS AND METHODS

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MATERIALS AND METHODS

Animal care. The care of all animals was approved by the Animal Care and Use Committee of Graduate School of Pharmaceutical Sciences, Osaka University. The investigation conforms to the *Guide*

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National Institutes of Health (NIH publication No. 85-23, revised 1996).

All mice for the experiments were euthanized by inhalation of isoflurane in a euthanasia chamber. Death of the animals was confirmed by monitoring the absence of breath after removal of the carcass from the euthanasia chamber. A total of 172 mice were used in this study.

I/R model and IL-11 treatment. Murine I/R was generated as described previously, with minor modifications (15, 21). Briefly, C57BL/6 mice (8- to 12 wk old; Japan SLC) were anesthetized and ventilated with 80% oxygen containing 1.5% isoflurane (Merck). After left-side thoracotomy, 7-0 silk suture was tied around the left coronary artery with a slipknot. Infarction was confirmed by discoloration of the ventricle and ST-T changes in electrocardiogram monitor. The chest and the skin were closed with 5-0 silk sutures. The mice were revived for a 30-min ischemic period, after which the knot was released and the heart was allowed to reperfuse for 24 h. By this experimental protocol, the mortality was minimized to less than 10%. Twenty four hours after reperfusion, the mice were euthanized and the slipknot was retied. PBS containing 1.5% Evans blue was injected into the left ventricle, and the hearts were removed. Isolated hearts were sectioned, and viable myocardium was stained with 2% triphenyl tetrazolium chloride (Sigma), as described previously (21). The amounts of myocardial area not at risk, area at risk (AAR), and infarcted area were quantified with Scion Image (Scion). In the IL-11 group, basically, 20 μg/kg of recombinant human IL-11 (Peprotech) was intravenously administered at the start of reperfusion (various concentrations in 200 μl of PBS/25 g of body wt), whereas the control group received the same volume of PBS over the same period. In the study concerning the dose-dependent effects of IL-11 on myocardial injury, various concentrations (3, 8, 20, 50 μg/kg) of IL-11 were used. There was no difference in mortality between groups.

Immunoblot analysis. Immunoblot analyses were performed as described previously (18). Briefly, heart homogenates were prepared in buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1% protease inhibitor cocktail, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 1 mM NaF. Proteins were separated by SDS-PAGE and transferred onto the polyvinylidene difluoride membrane (Millipore). The membrane was immunoblotted with anti-phospho-STAT3 (p-STAT3; Cell Signaling Technology) or anti-STAT3 (Santa Cruz Biotechnology) antibody. The membrane was reprobed with anti-STAT3 or anti-GAPDH (Santa Cruz Biotechnology) antibody to show the equal amount loading. Electrochemiluminescence system was used for the detection.

Echocardiographic analysis. Mice were exposed to I/R injury and IL-11 (20 μg/kg) or PBS, as a control, was administered at start of reperfusion. Twenty four hours after reperfusion, two-dimensional and motionmode (M-mode) transthoracic echocardiography was performed using an iE33 model equipped with a 15-MHz transducer (Philips Electronics, Andover, MA). Echocardiographic measurements were taken on M-mode. The investigator was blinded to the identity of the mice for analysis. Sham indicates the mice underwent thoracotomy without I/R.

Hemodynamic analysis. Hemodynamics was analyzed according to previous report with minor modification (20). Briefly, 24 h after reperfusion, mice were anesthetized (50 mg/kg pentobarbital) and heparinized (50 units) via intraperitoneal injection. The hearts were rapidly excised and placed in ice-cold modified Tyrode's solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.45 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose, and 5 HEPES (pH 7.4). The aorta was cannulated and retrogradely perfused at a constant pressure of 100 mmH_2O with Tyrode's solution bubbled with 80% oxygen at 37°C. Thus the experiments were performed at 37°C by immersing the heart in Tyrode's solution in a water-jacketed chamber. The hearts were paced at 420 beats/min. The fluid-filled balloon was inserted into the left ventricle to monitor cardiac function. The balloon was attached to a pressure transducer, which was coupled to a 4S PowerLab (AD Instruments). Left ventricular developed pressure and maximal and minimal change in pressure over time were measured.

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling staining. Twenty four hours after reperfusion, the frozen sections (5 μm thick) were prepared from the portion in the middle of the infarct zone. Apoptotic cell death was detected by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining with in situ apoptosis detection kit (TaKaRa). The sections were costained with anti-sarcomeric α-actinin (Sigma) antibody to identify the cardiomyocytes. Nuclei were simultaneously stained with Hoechst 33258. For quantitative analyses, apoptotic myocytes were counted in number by the researcher who was blinded to the assay conditions.

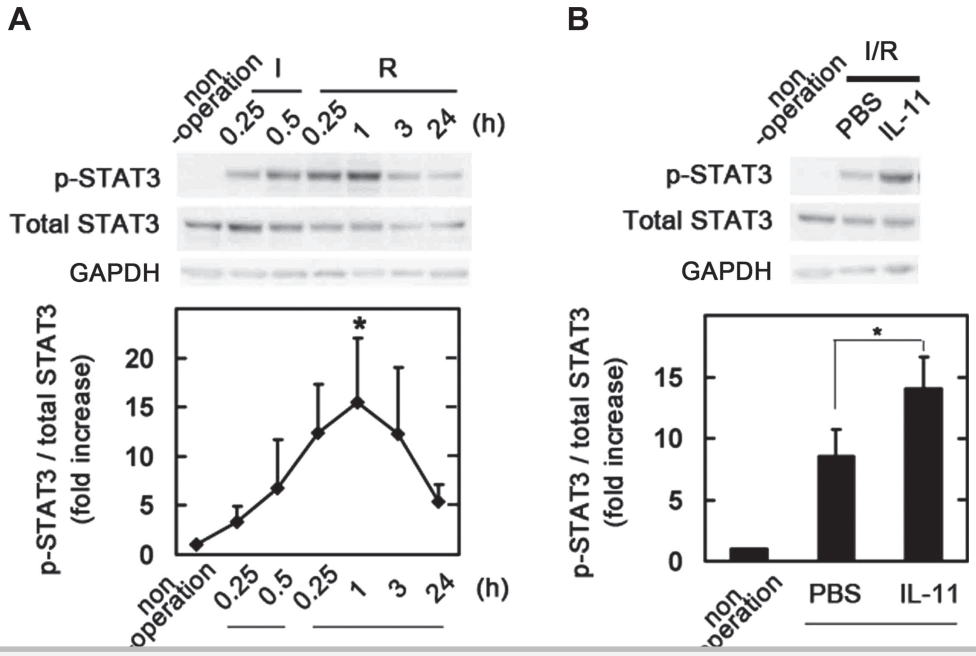

Fig. 1. IL-11 treatment enhanced STAT3 activation in ischemia/reperfusion (I/R) hearts. *A*: mice were exposed to I/R. At indicated time points, mice were euthanized and the lysates from hearts were immunoblotted with anti-phospho-specific STAT3 (p-STAT3) antibody. The blots were reprobed with anti-STAT3 antibody or GAPDH antibody. Representative data (*top*) and quantitative analyses of the p-STAT3 (*bottom*) are shown. Data are shown as means ± SD (n = 3 mice for each condition). *$P < 0.05$ vs. nonoperation, by 1-way ANOVA followed by Bonferroni test. *B*: mice were exposed to I/R. IL-11 (20 μg/kg) or PBS, as a control, was administered intravenously at start of reperfusion. Fifteen minutes after treatment, mice were euthanized and the lysates from hearts were immunoblotted with anti-p-STAT3 antibody. Blots were reprobed with anti-STAT3 antibody or GAPDH antibody. Representative data (*top*) and quantitative analyses of the p-STAT3 (*bottom*) are shown. Data are shown as means ± SD (n = 4 mice for each condition). *$P < 0.05$ vs. nonoperation, by 1-way ANOVA followed by Bonferroni test.

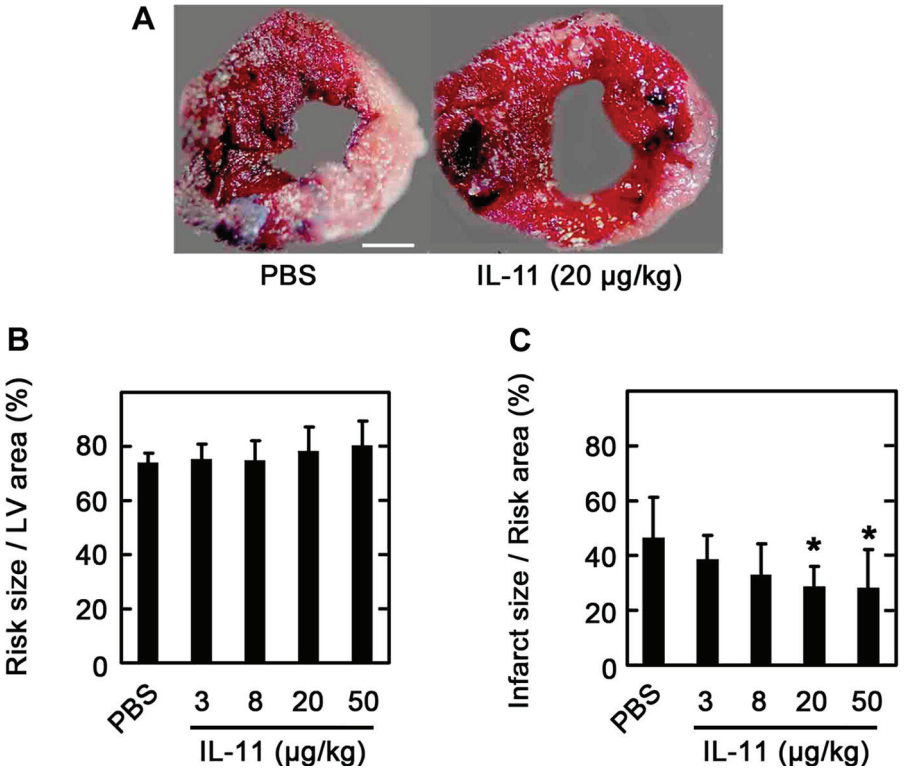

Fig. 2. Single administration of IL-11 at the time of reperfusion attenuated I/R injury. C57BL/6 mice were exposed to 30 min ischemia, followed by 24 h reperfusion. IL-11 or PBS, as a control, was administered intravenously at start of reperfusion. Areas at risk were estimated by exclusion of Evans blue. The myocardial infarct areas were detected by staining with 2% triphenyl tetrazolium chloride (TTC). Representative images are shown (*A*). Scale bar, 1 mm. *B* and *C*: risk area size and infarct size were quantitatively estimated. Data are shown as means ± SD (n = 9 mice for PBS; n = 6 mice for 3 μg/kg of IL-11; n = 7 mice for 8 μg/kg of IL-11; n = 9 mice for 20 μg/kg of IL-11; n = 6 mice for 50 μg/kg of IL-11). *P < 0.05 vs. PBS, by 1-way ANOVA followed by Bonferroni test. LV, left ventricular.

Dihydroethidium fluorescence analysis. Dihydroethidium (DHE) fluorescence analysis was performed to examine the generation of superoxide. The frozen sections (5 μm thick) were prepared and stained with 10 μM DHE in Krebs/HEPES buffer composed of (in mmol/l) 99.01 NaCl, 4.69 KCl, 1.87 $CaCl_2$, 1.20 $MgSO_4$, 1.03 K_2HPO_4, 25.0 $NaHCO_3$, 20.0 Na-HEPES, and 11.1 glucose (pH 7.4) at 37°C for 30 min in a dark. The intensities of fluorescence were quantitatively analyzed with Adobe Photoshop Elements 2.0 (Adobe Systems) by the researcher who was blinded to the assay conditions.

Real-time RT-PCR. Real time RT-PCR was performed according to the manufacturer's protocol. Total RNA was prepared from hearts at 3 h after reperfusion. Total RNA (1 μg) was subjected to first-strand cDNA synthesis with oligo (dT) primer. The mRNA expression was quantified by real-time RT-PCR using the Applied Biosystems StepOne Real-Time PCR systems (Applied Biosystems) with SYBR green system (Applied Biosystems). As an internal control, the expression of GAPDH mRNA was estimated with SYBR green system. The primers used in this study are as follows: metallothionein (MT) 1, forward: CGT AGC TCC AGC TTC ACC AGA TCT C, reverse: TGG TGG CAG CGC TGT TCG T; MT-2, forward: GCT TTT GCG CTC GAC CCA ATA CTC TC, reverse: GGA GCA GCA GCT TTT CTT GCA GGA AG; cyclooxygenase (COX)-2, forward: ACT GCC CAA CTC CCA TGG GT, reverse: AGT CCA CTC CAT GGC CCA GT; MnSOD, forward: AGG AGA GCA GCG GTC GTG TAA ACC T, reverse: CGG TGG CGT TGA GGT TGT TCA CGT A; Cu/ZnSOD, forward: AGA GCC TGA CAG GTG CAG AGA ACC, reverse: ACT TTG GCA TGC GTG TCG CC; redox factor-1, forward: AGA GAC CAA GAA GAG TAA GGG G, reverse: TGC TTC TTC CTT TAC CCA ATC C; peroxiredoxin 5, forward: TCA AGG TGG GAG ATG CCA TTC, reverse: AAC CTT GCC TTC TGC CTG GT; peroxiredoxin 6, forward: AGA TTC ATG GGG CAT TCT CTT TTC C, reverse: TAA GCA TTG ATG TCC TTG CTC CAG; isocitrate dehydrogenase, forward: AAG GAG AAG CTC ATC CTG CC, reverse: TCA GCT TGA ACT CTT CCA CAC G; glutathione reductase, forward: TGA TCA GGC ATG ATA AGG TAC TGA G, reverse: CAT CCG TCT GAA TGC CCA CT; glutathione peroxidase 4, forward: AGG CAG GAG CCA GGA AGT A, reverse: TGA TGG CAT TTC CCA GCA TGC; 5-oxoprolinase, forward: TTC CAG GGC CAG CTA AAG AAT G, reverse: TCT GTG GAT GTG CCT CCC ATG T; nuclear factor-like 1, forward: TGC ACA GTT CCC AGC TGA C, reverse: CTT CCA TAG CCT GCA TTT CCA T;

Table 1. *Effects of IL-11 on cardiac function at 24 h after reperfusion*

Parameter/Group	Sham	I/R + PBS	I/R + IL-11
Ejection fraction, %	79.3 ± 2.5	52.2 ± 5.7$	62.9 ± 10.1#*
Fractional shortening, %	40.8 ± 1.9	21.9 ± 3.1$	28.6 ± 6.2#*
Diastolic interventricular septal thickness, cm	0.085 ± 0.001	0.085 ± 0.012	0.093 ± 0.005
LV, cm			
Diastolic internal diameter	0.391 ± 0.033	0.360 ± 0.036	0.378 ± 0.009
Diastolic posterior wall thickness	0.069 ± 0.003	0.080 ± 0.012	0.077 ± 0.011
Systolic internal diameter	0.232 ± 0.026	0.282 ± 0.035	0.270 ± 0.028
Heart rate-LV, beats/min	494 ± 21	490 ± 52	486 ± 45

Values are means ± SD; n = 3 mice for sham, n = 6 mice for ischemia-reperfusion (I/R) + PBS, and n = 6 mice for I/R + IL-11. Mice were subjected to 30 min of ischemia followed by 24 h reperfusion. IL-11 (20 μg/kg) or PBS, as a control, was intravenously administered at the time of reperfusion. $$P$ <

Table 2. *Effects of IL-11 on hemodynamics at 24 h after reperfusion*

Parameter/Group	Sham	I/R + PBS	I/R + IL-11
LV developed pressure, mmHg	79.5 ± 9.9	55.6 ± 11.0$	69.3 ± 6.7*
+dP/d*t* (mmHg/s)	2347.8 ± 443.2	1532.4 ± 296.1$	2009.0 ± 321.2*
−dP/d*t* (mmHg/s)	−2178.3 ± 387.3	−1395.0 ± 326.1$	−1776.7 ± 132.2$*

Values are means ± SD; $n = 4$ mice for sham, $n = 5$ mice for I/R + PBS, and $n = 6$ mice for I/R + IL-11. Mice were subjected to 30 min of ischemia followed by 24 h reperfusion. IL-11 (20 μg/kg) or PBS, as a control, was intravenously administered at the time of reperfusion. $\$P < 0.05$ vs. Sham; $*P < 0.05$ vs. I/R + PBS, by unpaired *t*-test. ±dP/d*t*, maximal and minimal change in pressure over time.

GAPDH, forward: GCC GGT GCT GAG TAT GTC GT, reverse: CCC TTT TGG CTC CAC CCT T.

Cell culture and reagents. Cardiomyocytes were cultured as described previously (16). Briefly, cardiac ventricles of 1-day-old Wistar rats were minced and cells were isolated with 0.1% trypsin (Difco Laboratories) and 0.1% collagenase type IV (Sigma). To eliminate the nonmyocyte population, isolated cells were plated and incubated for 1 h at 37°C. Nonattached cells were collected as cardiomyocytes and cultured in DMEM/Ham's F-12 (DMEM/F-12) containing 5% neonatal calf serum. More than 90% cells were identified as cardiomyocytes, assessed by immunostaining with anti-sarcomeric specific α-actinin antibody.

STAT3 Stealth RNAi, MT Stealth RNAi, and control Stealth RNAi were purchased from Invitrogen. Cardiomyocytes were transfected with these small interfering RNA (siRNA) using Lipofectamine RNAi MAX (Invitrogen) in DMEM/F-12 containing 5% neonatal calf serum. Cardiomyocytes were cultured in serum-free DMEM/F-12 containing IL-11 and/or H_2O_2 at the indicated concentrations. Apoptotic cells were detected by Annexin V staining, as described in a previous report (16).

Conditional ablation of STAT3 gene in cardiomyocytes of adult murine hearts. Cardiac STAT3 conditional knockout mice were generated as described previously with minor modifications (20). In brief, the cardiac-specific transgenic mice overexpressing Cre recombinase fusion protein to the mutated estrogen receptor domains (MerCreMer) under the control of α-myosin heavy chain (α-MHC) promoter were crossed with STAT3 flox mice ($STAT3^{flox/flox}$) to produce α-MHC-MerCreMer/$STAT3^{flox/flox}$ mice. To activate Cre-recombinase activity, α-MHC-MerCreMer/$STAT3^{flox/flox}$ or α-MHC-MerCreMer/$STAT3^{wild/wild}$ mice, as control mice, were intraperitoneally injected with 8 mg/kg of tamoxifen (Sigma) dissolved in corn oil (Sigma) once a day for 14 consecutive days. After tamoxifen treatment, the mutant mice underwent I/R as described above.

Statistical analysis. Data were presented as means ± SD. The comparison between two groups was performed using an unpaired *t*-test. One-way ANOVA with Bonferroni test was used for comparisons of multiple groups. Differences were considered statistically significant when the calculated *P* value was less than 0.05.

RESULTS

IL-11 treatment enhanced STAT3 activity in I/R hearts. Because it is known that STAT3 is activated during I/R, we first confirmed that STAT3 is endogenously activated in hearts at various time points after I/R in our system (Fig. 1*A*). Immunoblot analyses with anti-p-STAT3 antibody revealed that STAT3 phosphorylation was slightly induced at a 30-min ischemia period. It is important that STAT3 was dramatically activated at 1 h after reperfusion. These data indicated that STAT3 signals are endogenously activated during I/R.

Next, we examined whether IL-11 administration further enhanced STAT3 activity during I/R (Fig. 1*B*). Mice were exposed to I/R injury with intravenous injection of IL-11 at a dose of 20 μg/kg or PBS at start of reperfusion. We analyzed the activation of STAT3 at 15 min after reperfusion by immunoblot analysis, based on the previous findings that the intravenous administration of IL-11 activated STAT3 with its peak at 15 min in nonoperated hearts (20). Fifteen minutes after reperfusion, I/R stimuli induced STAT3 activation, which was enhanced by IL-11 treatment, relative to control.

The therapeutic treatment of IL-11 exhibits the postconditioning effects against I/R injury. Because STAT3 activity was reinforced by IL-11 injection at start of reperfusion in I/R

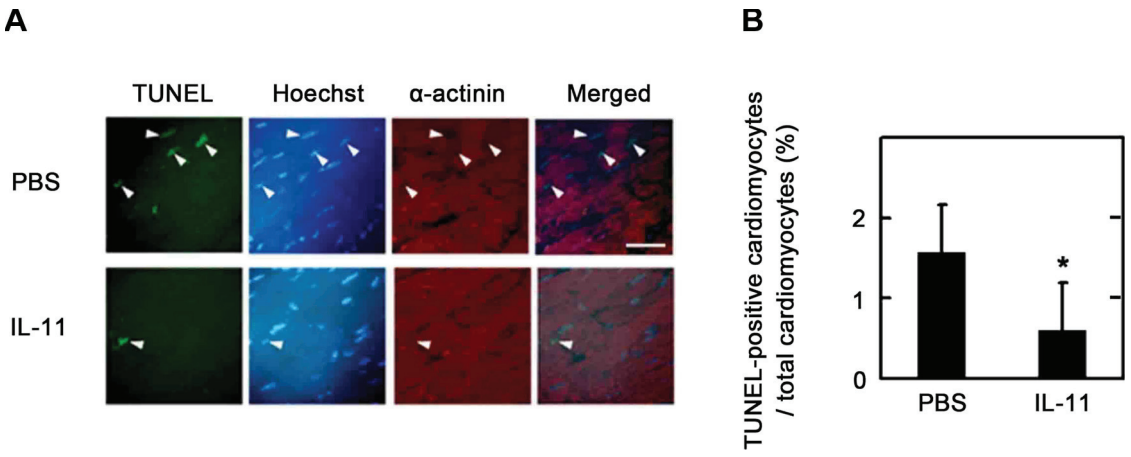

Fig. 3. IL-11 treatment suppressed cardiomyocytes apoptosis after I/R. *A*: frequency of apoptotic cardiomyocytes was estimated by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining 24 h after myocardial infarction. The sections were costained with antisarcomeric α-actinin antibody and Hoechst 33258 dye. The images shown are representative of 75 to 120 images obtained from 5 to 6 mice (15 to 20 fields from each mouse). Arrowheads show TUNEL-positive, apoptotic cardiomyocytes. Scale bar, 50 μm. *B*: quantification of the apoptotic cardiomyocytes is shown. Data are shown

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