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Interleukin-27, a novel cytokine induced by ischemia–reperfusion injury in rat hearts, mediates cardioprotective effects via the gp130/STAT3 pathway

Ming-Chieh Ma[1] · Bao-Wei Wang[2] · Tzu-Pei Yeh[1] · Jia-Long Wu[1] ·
Tun-Hui Chung[1] · Kochung Tsui[3] · Chih-Fan Chiang[1] · Ai-Ju Huang[1] ·
Yu-Tzu Huang[1]

Abstract Patients with coronary artery disease show high serum levels of interleukin (IL)-27, a novel member of the IL-6 family. However, the function of IL-27 in hearts suffering ischemia/reperfusion (IR) injury is unclear. Here, we showed increased expression of mRNA for the IL-27 subunits, EBI3 and p28, in rat hearts after 40 min of coronary ligation and release for 7 days. This increase was associated with a peak in the release of the cardiac enzyme, creatine kinase-MB, on day 2 post-release. Moreover, levels of IL-27 receptor subunit gp130 mRNA, but not those of subunit WSX-1 mRNA, decreased in post-is-chemic hearts. These results suggest that increased IL-27 production may compensate for receptor downregulation during myocardial recovery. Lactate dehydrogenase release and crystal violet staining revealed that IL-27 or IL-6 significantly attenuated severe hypoxia (SH, 2 % O$_2$)-in-duced cell damage in H9c2 cardiomyoblasts and primary rat neonatal cardiomyocytes. Incubating cardiomyocytes with IL-27 or IL-6 resulted in time-dependent activation of signal transducers and activators of transcription 3 (STAT3). Interestingly, IL-27-induced STAT3 activation was attenuated by pre-treatment with a gp130-neutralizing antibody. Blocking gp130 also reduced the cytoprotective effects of IL-27 or IL-6. Moreover, IL-27-mediated protection against SH was blocked by stattic, a small-molecule inhibitor of STAT3. IL-27 markedly improved post-ischemic recovery and reduced tissue damage in iso-lated perfused hearts when administered 5 min before reperfusion. These results indicate that IL-27 protects the myocardium against IR injury and facilitates the recovery of damaged cardiomyocytes via the gp130/STAT3 pathway.

Keywords IL-27 · Cardiomyocytes · Hypoxia · Ischemia/reperfusion · Cell death · gp130 · STAT3

Introduction

Ischemia, either acute or chronic, is often associated with tissue injury and inflammation, which can lead to ischemic cardiomyopathy [12, 31]. Previous studies in genetically modified animal models show that excessive production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, contributes to the tissue damage associated with heart failure [25, 26]. However, recent studies report that these cytokines may promote activation of the pro-survival molecule, signal transducer, and activator of transcription 3 (STAT3) to provide car-dioprotective effects. Under survival conditions, STAT3 acts as an essential component of the survivor activating factor enhancement (SAFE) signaling pathway and, as such, reduces cardiomyocyte death at the time of reperfu-sion after ischemic insult [1, 28]. STAT3 activation, trig-gered by the Janus kinase signaling pathway, has both anti-apoptotic and proliferative effects; indeed, activation of STAT3 in cardiomyocytes protects them from ischemia/reperfusion (IR) or anoxia/reoxygenation injury [2, 12–16, 22, 33, 35].

✉ Yu-Tzu Huang
med0043@mail.fju.edu.tw

1 School of Medicine, Fu Jen Catholic University,
510 Chungcheng Road, Hsinchuang District,
New Taipei 24205, Taiwan

2 Department of Education and Research, Shin-Kong Wu
Ho-Su Memorial Hospital, Taipei, Taiwan

3 Department of Clinical Pathology, Cathay General Hospital,
Taipei, Taiwan

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A previous study shows that IL-6 levels increase in patients with congestive heart failure and that the cytokine is released into the circulation from the border zones around a myocardial infarct [21]. Ischemic preconditioning protects the heart against IR injury; however, this phenomenon is not observed in IL-6 knockout mice with large infarcts caused by IR [8]. Interestingly, IR-induced apoptosis of cardiomyocytes is attenuated by treatment with IL-6 [32, 41], suggesting that the cytokine has a cardioprotective role during IR-related events. It is unclear whether IL-27 also protects against IR injury. Recently, a clinical study detected high levels of serum IL-27, a novel member of the IL-6 cytokine family, in patients with coronary artery disease [20]. IL-27 is a heterodimer comprising the Epstein–Barr viral-induced gene 3 (EBI3) and p28 [10, 37]. The major proportion of IL-27 is secreted by activated antigen-presenting cells and endothelial cells [48]. The IL-27 receptor was recently cloned and found to comprise a heterodimer of WSX-1 and transmembrane glycoprotein 130 (gp130) [36]. Gp130 is a common signal transducer for the IL-6 family, a group of cytokines displaying both unique and overlapping biological activities in multiple hematopoietic lineages. Activation of gp130 negatively regulates myocardial apoptosis [46], and abrogation of gp130 function exacerbates cardiac damage associated with pressure overload-induced heart failure [18].

Activation of gp130/STAT3 by cytokines and growth factors usually triggers cell proliferation, differentiation, and survival. We recently showed that IL-27 activates cord blood natural killer cells via the gp130/STAT3 pathway [5]. Therefore, the aim of the present study was to examine whether IL-27 exerts a cardioprotective effect via the gp130/STAT3 pathway. We found increased expression of IL-27 in the cardiac tissues of rat hearts following coronary ligation-induced IR injury in vivo. The cytoprotective properties of IL-27 and IL-6 were then examined in in vitro cardiomyocyte cultures. The IL-27-mediated effects were further assessed in terms of the gp130/STAT3 signaling pathway by blocking gp130 and STAT3 following exposure of cells to severe hypoxia (SH). Taken together, the results show for the first time that IL-27 protects rat hearts against IR injury.

Materials and methods

Animals

Adult male and pregnant female Wistar rats were obtained from BioLASCO (Taipei, Taiwan). Rats (210–230 g) were used for the IR and isolated perfused heart experiments. Primary cardiomyocyte cultures were prepared from 1- to 3-day-old neonatal rats. The preparation of neonatal cardiomyocyte cultures and animal experiments and care were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). All protocols were approved by the Laboratory Animal Care Committee at Fu Jen Catholic University.

Induction of acute myocardial infarction in rats as an in vivo model

Rats were anesthetized by an intraperitoneal injection of ketamine (60 mg/kg) and sodium pentobarbital (35 mg/kg). The rats were then intubated and ventilated with room air (60 breaths/min; tidal volume, 8 ml/kg). The rectal temperature was maintained at 37 °C with a servo-null heating pad. Left thoracotomy was performed aseptically and the left anterior descending artery looped with 7-O Prolene (Ethicon Inc., Somerville, NJ, USA) close to its origin (about 3 mm away from the left coronary ostium), as described previously [29]. The looped stitch was snared for 40 min and then released. Ischemia was confirmed by the appearance of regional cyanosis within the epicardium distal to the ligation. In addition, akinesia or bulging in the area was observed. The thorax was then closed aseptically. To measure creatine kinase-MB (CK-MB), the rats were anesthetized with sodium pentobarbital (60 mg/kg), and 200 μl of blood was collected from the abdominal aorta into a heparinized tube for analyses of cardiac enzymes. The blood was then centrifuged at $620g$ and the plasma collected. The activity of CK-MB was measured in an electrolyte analyzer (Dri-Chem 3500i, Fujifilm, Tokyo, Japan) as previously described [29]. Then, the tissues from infarct area or the corresponding area in the left ventricle were prepared for PCR analysis.

Preparation of primary neonatal rat cardiomyocytes

Neonatal cardiomyocyte cultures were prepared from 1- to 3-day-old Wistar rats as previously described [40]. Briefly, cardiomyocytes were obtained from neonatal hearts by digestion with 0.05 % trypsin. Cells (7×10^5 cells/well) were plated in 12-well plates and cultured in F10 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 100 μM of bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) (Sigma-Aldrich, Saint Louis, MO, USA), 1 % L-glutamine (Hyclone), and PSA (100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin B) (Biological Industries, Haemek, Israel). The contractile characteristics of the cultured cardiomyocytes, as observed under a light microscope, demonstrated a purity of more than 95 %. Cultures of enriched cardiomyocytes were maintained for 3 days at 37 °C in the presence of 5 % CO_2.

Culture of cardiomyocyte-like H9c2 cells

The H9c2 cardiomyoblast cell line was established from rat heart [23] and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Life Technologies), 1 % L-glutamine (GE Healthcare, Grand Island, Nebraska), and PSA (Biological Industries), as previously described [29].

Preparation of IL-27 and IL-6

IL-6, EBI3, or p28 cDNAs were cloned into pRK5F, which contains a CMV promoter-derived expression plasmid and a FLAG epitope at the C-terminus. The IL-6 gene was derived from phytohemagglutinin-stimulated human peripheral blood mononuclear cells. The EBI3 gene was amplified from a human placental cDNA library. The p28 gene was derived from an embryonic stem cell clone. The constructed expression plasmids were then transfected into the 293T human embryonic kidney cell line (BCRC Strain; Administration System, Shichu, Taiwan) as described previously [5]. Transfected cells were cultured in DMEM supplemented with 2 % fetal bovine serum plus antibiotics for 48 h and the culture supernatant was analyzed for the presence of IL-6, EBI3, and p28 by Western blot analysis. Rat primary neonatal cardiomyocytes or H9c2 cells were incubated with culture medium containing IL-6 or IL-27 for 6 h before hypoxic insult. Furthermore, for use in the assay involving isolated perfused hearts, culture medium containing IL-6 or IL-27 was concentrated tenfold using a Millipore Centricon plus-70 10 K centrifugal filter (Millipore, Bedford, MA, USA) and then diluted with ice-cold KH buffer.

Recombinant human IL-27 and IL-6 (R&D Systems, Minneapolis, MN, USA) were diluted in culture medium. Based on the molecular weights, the working concentrations were 150 and 60 ng/ml for IL-27 and IL-6, respectively.

Induction of severe hypoxia in cultured cells

SH was induced by incubating primary cardiomyocytes or H9c2 cells at 37 °C with 5 % CO_2 in an oxygen incubator constantly flushed with nitrogen to reduce the oxygen content to 2 % (NuAire, Plymouth, MN, USA) for 72 h. Control cells were incubated in a CO_2 incubator (NuAire) constantly flushed with room air (21 % oxygen) (normoxic conditions).

Evaluation of cytotoxicity and cell density

A cytotoxicity kit (Roche Applied Science, Mannheim, Germany) was used to measure lactate dehydrogenase (LDH) release into the cell culture supernatant. The absorbance of the dye was measured at 492 nm in a standard ELISA plate reader. The concentration of LDH was quantitated against an LDH standard (Sigma-Aldrich), as previously described [30]. Cells (7×10^5 cells/well) plated in 12-well plates were fixed with 10 % methanol for 10 min and then stained with 1 % crystal violet in PBS (pH 7.4) for 5 min. Cells were washed five times with PBS and the level of staining was measured to evaluate cell density.

Detection of IL-27 subunit and IL-27 receptor subunit expression by RT-PCR

The RNA Bee reagent (Tel-Test, Friendswood, Texas) was used to extract total RNA from rat neonatal cardiomyocytes, H9c2 cells, and the left ventricle of rat hearts at 2, 3, or 7 days post-IR injury, according to the manufacturer's instructions. Reverse transcription was performed using 1 μg of RNA, random primers, and M-MLV reverse transcriptase (Promega Corporation, Madison, Wisconsin). The resulting cDNA product (1 μl) was used in a PCR reaction along with primers specific for EBI3, p28, gp130, or WSX-

Table 1 Nucleotide sequences of the primers used for RT-PCR and quantitative RT-PCR

Target gene (size of the PCR product)	Nucleotide sequence
EBI3 (215 bp)	Forward, 5′-GTCCTTCATTGCCACKTACAGGCT-3′
	Reverse, 5′-GGAGGGTCSGGCTTGATGAT-3′
P28 (154 bp)	Forward, 5′-AGGGARTTCACAGTCAGCCTG-3′
	Reverse, 5′-GCCTGGAARGTCAGGGAAACAT-3′
gp130 (163 bp)	Forward, 5′-ATACCTTAAACAAGCTCCACCTTC-3′
	Reverse, 5′-AGTTTCATTTCCAATGATGGTTCT-3′
WSX-1 (155 bp)	Forward, 5′-GAAACCCAAATGAAGCCAAA-3′
	Reverse, 5′-GCCTCCTGACATCTTCGGTA-3′
GAPDH (108 bp)	Forward, 5′-TTGGTATCGTGGAAGGACTC-3′
	Reverse, 5′-CAGTAGAGGCAGGGATGAT-3′

1 (400 nM each of sense and anti-sense primers), dNTPs (200 mM), 1 × PCR reaction buffer, and ProZyme II thermostable DNA polymerase (Protech Technology Enterprise, Taipei, Taiwan). The primer sequences are listed in the Table 1. The cycling conditions for 30 cycles were as follows: 94 °C for 40 s, 58 °C for 20 s, and 72 °C for 40 s. PCR products were analyzed on 1.6 % agarose gels and visualized by staining with ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control.

Real-time PCR

Real-time PCR was performed in an ABI StepOne Plus system (Applied Biosystems Foster City, CA, USA). The reaction mixture (20 μL total volume) comprised 200 ng of cDNA, 30 μmol of each primer (Table 1), and Sybr Green (PCR master mix kit; Applied Biosystems). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. Melting curve analysis was performed at the end of each PCR experiment. All reactions were run in duplicate. Relative changes in gene expression were calculated using the ΔC_t (threshold cycle) method, i.e., the raw C_t value of the house-keeping gene (GAPDH) was subtracted from the raw C_t value of the target gene. Changes in target gene expression were calculated using the formula $2^{-\Delta C_t}$ and expressed as the fold change relative to the values in control hearts.

Neutralizing antibodies and pharmacological inhibitors

A gp130-neutralizing antibody (C-20) was raised in a rabbit (Santa Cruz Biotechnology, Paso Robles, CA, USA). Cardiomyocytes and cell lines were incubated with the STAT3 inhibitor, stattic (6-nitrobenzo[b]thiophene 1,1-dioxide; Merck, Darmstadt, Germany), dissolved in dimethyl sulfoxide (DMSO). The same concentration of DMSO was used as a vehicle control.

Western blot analysis of STAT3 expression

To examine the expression of phospho-STAT3 and total STAT3, cells were washed with PBS and harvested by incubation in lysis buffer containing 0.6 % NP-40, 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 0.1 % sodium dodecyl sulfate for 5 min as previously described [29, 30]. Cells were sonicated on ice for 2 min and the lysates were collected by centrifugation at 14,000g for 10 min. Next, 40 μg of cell lysate was resolved on a 10 % sodium dodecyl sulfate–polyacrylamide gel (Bionovus, Ontario, Canada) and the proteins were transferred to a nitrocellulose membrane. The membrane was incubated with antibody against phosphorylated STAT3, total STAT3, or β-actin (Cell Signaling Technology, Danvers, MA, USA), followed by washing and incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (Millipore). Proteins were visualized using an enhanced chemiluminescence kit (Millipore). The densities of the bands of appropriate molecular masses were determined semi-quantitatively by densitometry using an image analytic system (Diagnostic Instruments, Sterling Heights, MI, USA). STAT3 activity was calculated as the ratio of phospho-STAT3 to total STAT3 after normalized with β-actin.

Preparation of isolated perfused hearts as an ex vivo model

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After abdominal incision, 1 ml of heparin (500 IU) was injected via the inferior vena cava. The hearts were then rapidly excised, weighed, and immersed in ice-cold KH buffer, as previously described [29, 30]. After a 30 min non-circulating perfusion to remove blood cells, the following parameters were recorded continuously and averaged over 5 min: coronary perfusion pressure (CPP) at the aorta, left ventricular developed pressure (LVDP; measured by inserting a water-filled latex balloon), and the coronary flow (CF). Recirculation perfusion was performed with KH buffer (total volume, 100 ml). After a 20 min basal period, perfusion was stopped for 40 min to induce global ischemia. Hearts were then reperfused for 1 h. At 5 min before reperfusion, 10 ml of concentrated culture medium containing pRK5F (controls) or IL-27 was added to the buffer reservoir (after removing the same volume of KH buffer). At the end of experiment, 2 ml of perfusate was collected and concentrated to measure the level of CK-MB.

Statistical analysis

Numerical data are expressed as the mean ± the standard error of the mean (SEM). Differences between groups and time points were analyzed using two-way ANOVA with a post hoc Sidak's test. The levels of CK-MB in plasma were compared between pRK5F-treated and IL-27-treated IR hearts by Student t test. Differences were regarded as significant at $p < 0.05$.

Fig. 1 Changes in the expression of IL-27 mRNA and in the levels of ▶
cardiac enzymes in rat hearts subjected to IR insult. **a** The expression
of EBI3 and p28 mRNA in one or three hearts at days 2 (2 days), 3
(3 days), and 7 (7 days) post-IR was examined by RT-PCR.
Representative gels are shown. Cells transfected with expression
vectors containing either the EBI3 or p28 genes were used as positive
controls (+) and cells transfected with empty vectors were used as
negative controls (−). GAPDH was used as an internal control. **b**,
c Real-time quantitative RT-PCR was used to examine fold changes
of EBI3 (**b**) and p28 (**c**) mRNA expression in control (**c**, sham-
operated) and IR hearts at 2, 3, and 7 days post-IR induction. ΔC_T
values were calculated to estimate relative changes in gene expression
after subtracting the values for GADPH expression ($n = 6$ per time
point). **d** Increased levels of creatine kinase (CK)-MB in the plasma at
0, 1, 2, 3, and 7 days post-IR confirmed myocardial damage ($n = 6$
for each time point). $*p < 0.05$, compared with day 0; $^{\dagger}p < 0.05$, IR
vs. control (sham) hearts

Results

IL-27 is upregulated in post-ischemic hearts

Conventional PCR detected the expression of EBI3 mRNA
in the post-ischemic left ventricle from day 2 to day 7 after
hypoxic insult (Fig. 1a). Quantitative RT-PCR revealed
that EBI3 expression was 2.6- and 1.8-fold higher than that
in control hearts on days 2 and 3 post-IR, but returned to
control levels by day 7 (Fig. 1b). Conventional PCR de-
tected p28 mRNA expression in IR hearts on day 3 post-
insult (Fig. 1a). Quantitative RT-PCR revealed that p28
mRNA was 1.5- and 1.9-fold higher than that in control
hearts on days 3 and 7 in IR hearts, respectively (Fig. 1c).
Increases in IL-27 mRNA levels were associated with in-
creased release of cardiac enzymes (Fig. 1d). Compared
with pre-ligation levels (day 0), plasma levels of CK-MB
increased in IR rats, peaking on day 2 before falling again.
Plasma levels of CK-MB were also higher in IR rats than in
sham-operated rats at comparable time points. Taken to-
gether, these results suggest that IL-27 may play a dual role
in post-ischemic injury, possibly by protecting the my-
ocardium against IR injury or aggravates cardiac damage
during recovery.

Expression of gp130 and WSX-1 mRNA

A previous study showed that the IL-27 receptor is
heterodimeric, comprising gp130 and WSX-1 [36].
Therefore, we next examined the expression of gp130 and
WSX-1 mRNA in the myocardium and in cardiomyocyte-
like cells. Expression of gp130 and WSX-1 mRNA was
detectable in neonatal cardiomyocytes, in the left ventricle
of the rat heart, and in cardiomyocyte-like H9c2 cells
at baseline (Fig. 2a); however, expression in neonatal
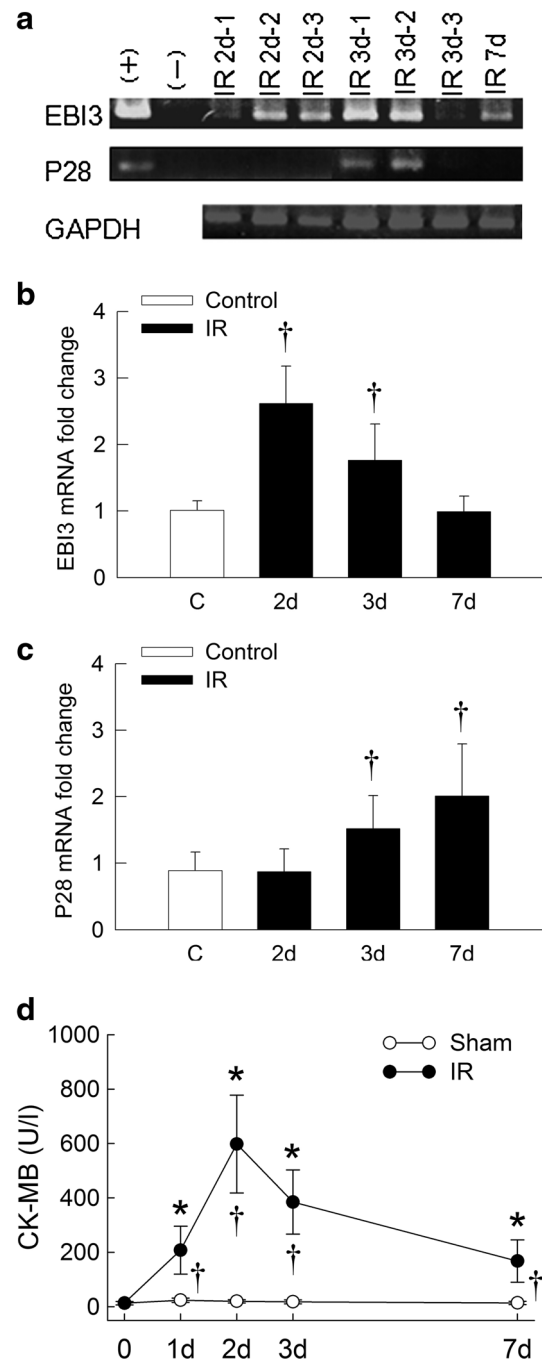
cardiomyocytes and cardiac tissue was higher than that in
H9c2 cells. We next used quantitative RT-PCR to examine
changes in gp130 and WSX-1 mRNA expression under
stress conditions, i.e., post-IR treatment, and found a sig-
nificant reduction in gp130 mRNA expression at days 2–7
after IR (both $p < 0.05$, Fig. 2b) compared with that in
control hearts. However, there was no difference in the
expression of WSX-1 mRNA (Fig. 2c).

IL-27 protects neonatal cardiomyocytes against SH and increases STAT3 activity

Because rat neonatal cardiomyocytes express IL-27 receptors, we next asked whether IL-27 protects cardiomyocytes against SH (a process that would mimic hypoxic injury to the myocardium during ischemic insult in vivo). The results showed that cells subjected to SH released more LDH than cells incubated under normoxic conditions; this increase peaked at 72 h post-SH (Fig. 3a).

Because IL-6-mediated STAT3 activation is thought to be cytoprotective [41], we compared the effects of IL-27 with those of IL-6 with respect to cardioprotection. Prior treatment of neonatal cardiomyocytes with IL-27 or IL-6 for 6 h led to a significant increase in cell viability (upper panel; Fig. 3b) and a reduction in LDH release after exposure to SH for 72 h (Fig. 3b). These results indicate that IL-27 attenuates SH-induced cell damage. Interestingly, IL-6 showed similar protective effects against SH.

Because increased STAT3 activity protects cells against numerous cytotoxic effects [2, 35], we next examined whether STAT3 in neonatal cardiomyocytes was activated by IL-27 or IL-6. Compared with cells treated with the control vector, pRK5F, exposure of neonatal cardiomyocytes to either IL-27 or IL-6 for 0.5, 1, or 2 h led to increased expression of phosphorylated STAT3 (p-STAT3; upper blots in Fig. 3c) and increased STAT3 activity (bar graph in Fig. 3c).

IL-27 protects H9c2 cells against SH-mediated cell damage and activates STAT3

To further confirm that IL-27 protects cells against SH, we examined the effects of IL-27 in cardiomyocyte-like H9c2 cells expressing IL-27 receptors. Compared with cells incubated under normoxic conditions or cells at time point 0 (prior to SH induction), cells exposed to SH showed a significant increase in LDH release between 48 and 96 h (Fig. 4a). Similarly, at 72 h post-SH, higher cell viability and lower LDH release were seen in cells pretreated with IL-27 or IL-6 than those in untreated cells or cells harboring pRK5F (Fig. 4b). Moreover, treatment of H9c2 cells with IL-27 or IL-6 for 2 h increased STAT3 phosphorylation and activity (Fig. 4c).

Blocking gp130 abolishes the effects of IL-27

To further confirm the cardioprotective role of IL-27, we treated H9c2 cells with recombinant human IL-27 or IL-6 and measured LDH release after SH. Compared with normoxic cells, treatment of H9c2 cells with recombinant IL-27 or IL-6 led to a marked reduction in LDH release at 72 h post-SH (Fig. 5a). Interestingly, these effects were attenuated
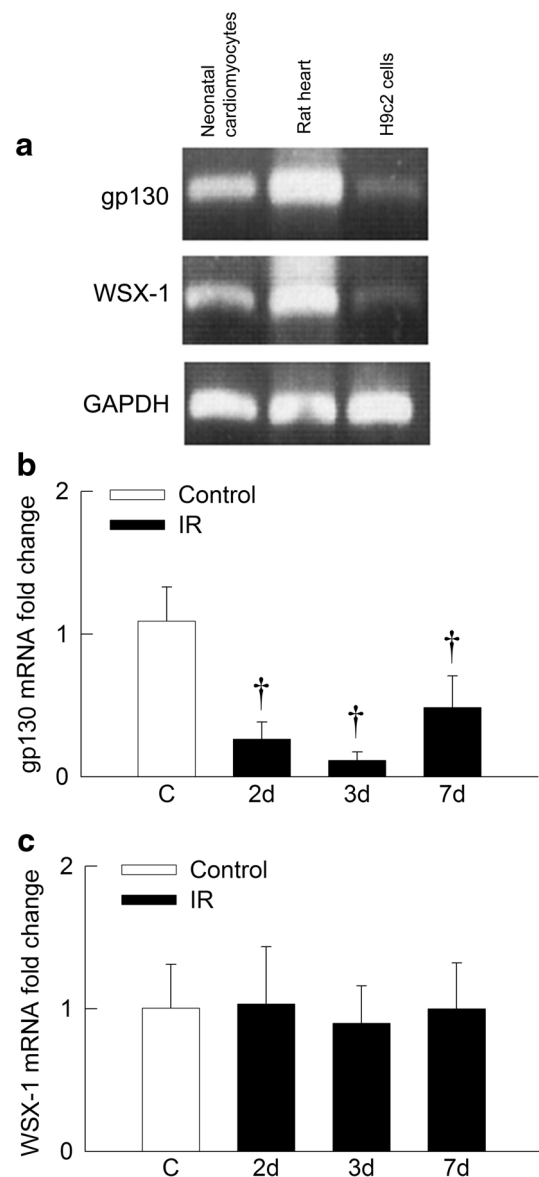
Fig. 2 Expression of IL-27 receptor subunit mRNA. **a** Expression of gp130 and WSX-1 mRNA in neonatal eat cardiomyocytes, rat heart tissue, and cardiomyocyte-like H9c2 cells. Representative images are shown. GAPDH was used as a control. Fold changes in gp130 (**b**) and WSX-1 (**c**) mRNA expression in control (c) and IR hearts at 2 (2 days), 3 (3 days), and 7 (7 days) days post-IR ($n = 6$ per time point). $^{\dagger}p < 0.05$, IR vs. control hearts

by co-treatment with a neutralizing antibody specific for gp130 (Fig. 5b). This clearly indicates that the cardioprotective effects of IL-27 and IL-6 are dependent on gp130.

The effects of the gp130-neutralizing antibody were further confirmed in cells treated with culture medium containing recombinant IL-27 or IL-6. Crystal violet staining showed that the anti-gp130 antibody reduced the adhesion of both neonatal cardiomyocytes and H9c2 cells; this was not observed upon treatment with a control antibody (upper panels in Fig. 5c, d). In addition, the gp130-
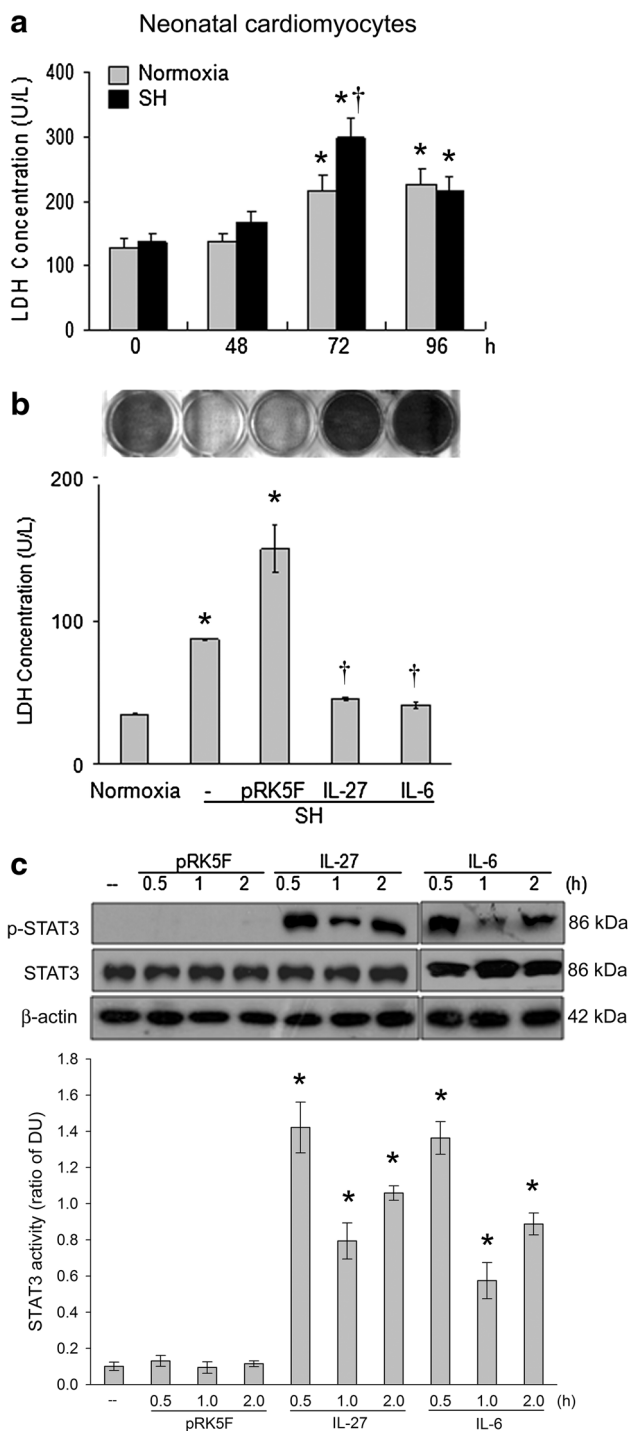
a Neonatal cardiomyocytes

b

c

neutralizing partially reversed the IL-27/IL-6-mediated reduction in LDH release (lower panels in Fig. 5c, d).

The gp130-neutralizing antibody attenuates IL-27-mediated STAT3 activation

We next examined whether blocking gp130 affected IL-27-mediated signal transduction mediated by STAT3. In H9c2 cells, treatment of gp130-neutralizing antibody in control

◄**Fig. 3** IL-27 attenuates SH-mediated cytotoxicity and increases STAT3 activity in neonatal rat cardiomyocytes. **a** Cells were exposed to either normoxia (21 % O_2) or severe hypoxia (SH, 2 % O_2) for 0, 48, 72, or 96 h. LDH release ($n = 5$) was then measured to estimate cell viability. *$p < 0.05$, compared with 0 h; †$p < 0.05$, SH vs. normoxia at 72 h. **b** Cells were treated with either IL-27 or IL-6 for 6 h prior to exposure to SH. The *upper panel* shows representative culture wells stained with crystal violet (1 %) at 72 h post-SH induction. The *lower panel* shows LDH release ($n = 5$). *$p < 0.05$, untreated (−) or pRK5F vector-treated cells subjected to SH vs. normoxic conditions; †$p < 0.05$, IL-27- or IL-6-treated vs. untreated cells subjected to SH. **c** Representative immunoblots showing the expression of phosphorylated STAT3 (p-STAT3), total STAT3, and β-actin in cells treated with either IL-27 or IL-6 for 0.5, 1, and 2 h (*upper panel*). *Bar graphs* show the STAT3 activity ($n = 3$). *DU* density unit. *$p < 0.05$, IL-27- or IL-6-treated cells exposed to SH vs. pRK5F vector-treated cells exposed to SH

cells showed no effect on STAT3 activity when compared to untreated control cells (Fig. 6a). Treatment with culture medium containing IL-27 led to a significant increase in STAT3 phosphorylation and activity in H9c2 cells (Fig. 6a). This increase, however, was attenuated by co-treatment with the gp130-neutralizing antibody.

The cytoprotective effects of IL-27 against SH in H9c2 cells (as manifest by reduced LDH release) were abrogated by the STAT3 inhibitor, stattic, in a dose-dependent manner (Fig. 6b). The effects were only partial at a dose of 2 μmol/L stattic; however, complete reversal was observed at 10 μmol/L (compared with control (SH + pRK5F) cells). These data confirm that the cardioprotective effects of IL-27 require STAT3 activation.

IL-27 protects hearts against acute IR in an ex vivo perfused model

Since IL-27 protects cardiomyocytes against SH in vitro, we next asked whether IL-27 protects rat hearts against IR insult. We used an ex vivo perfusion model for this to prevent the results being confounded by IL-27 released by other circulatory cells. The aortic pressure was maintained at around 100 mmHg throughout the experiment. We found that the LVDP and CF rates were significantly lower in IR hearts throughout the entire time course of reperfusion (Fig. 7a). The contractile function of IL-27-treated hearts was stronger than that of pRK5F-treated hearts throughout the experiment. Improved cardiac function was accompanied by a reduction in CK-MB release (49 ± 7 % in IL-27-treated hearts vs. 100 % in pRK5F-treated hearts), suggesting less myocardial damage in IR hearts treated with IL-27 (Fig. 7b).

Discussion

The results of the present study show that IL-27 and its receptor are expressed in rat cardiac tissue, although they were upregulated and downregulated, respectively, in post-
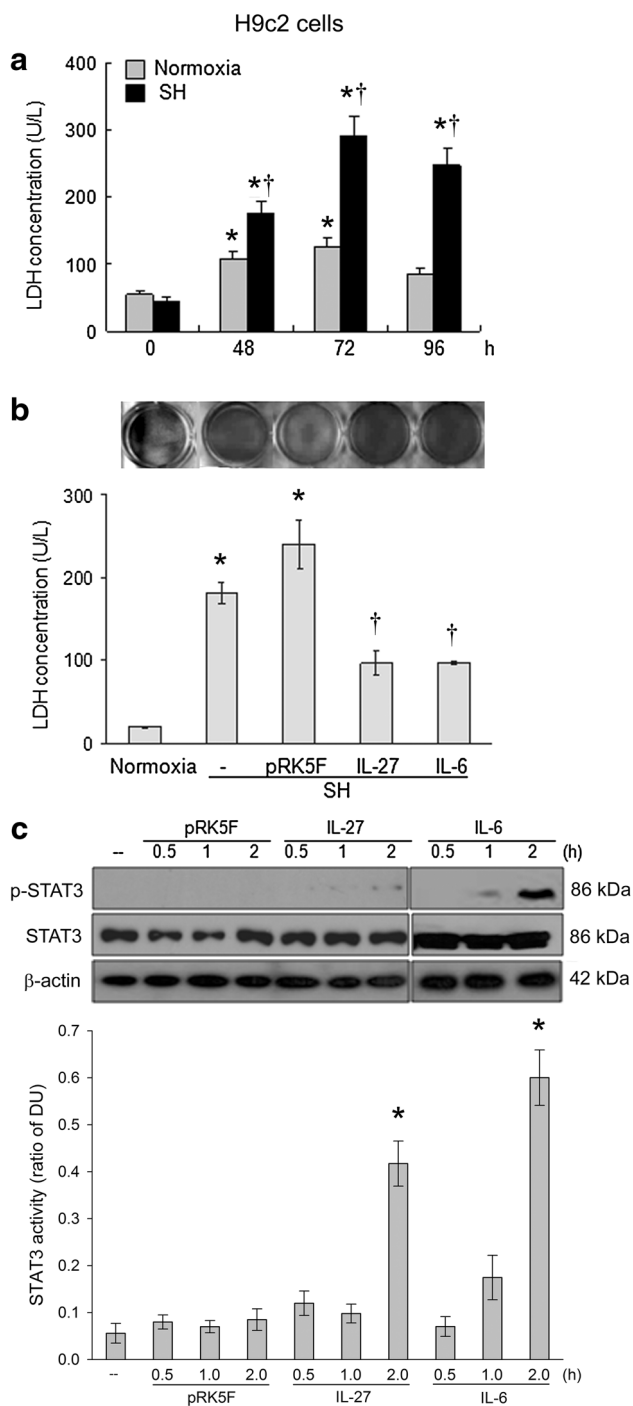
H9c2 cells

a

b

c

◄ **Fig. 4** IL-27 attenuates SH-mediated cytotoxicity and increases STAT3 activity in H9c2 cells. **a** Cells were exposed to either normoxia (21 % O_2) or severe hypoxia (SH, 2 % O_2) for 0, 48, 72, or 96 h, and LDH release ($n = 5$) was measured to estimate cell viability. *$p < 0.05$, compared with 0 h; †$p < 0.05$, SH vs. normoxic conditions. **b** Cells were treated with either IL-27 or IL-6 for 6 h prior to exposure to SH for 72 h. *$p < 0.05$, untreated (−), or pRK5F vector-treated cells exposed to SH vs. normoxic conditions; †$p < 0.05$, IL-27- or IL-6-treated cells exposed to SH vs. untreated cells exposed to SH. *Upper panel* shows representative culture wells stained with crystal violet (1 %) to evaluate cytotoxicity at 3 days after the induction of SH. *Lower panel* shows LDH release ($n = 5$). **c** Representative immunoblots showing the expression of phosphorylated STAT3 (p-STAT3), total STAT3, and β-actin in cells treated with either IL-27 or IL-6 for 0.5, 1, and 2 h (*upper panel*). *Bar graphs* show the STAT3 activity ($n = 3$). *DU* density unit. *$p < 0.05$, IL-27- or IL-6-treated cells under SH conditions vs. pRK5F vector-treated cells under SH conditions

but also abrogated IL-27-mediated cardioprotection. Blockade of STAT3 activity by stattic also reversed the IL-27-mediated protective effects. Finally, studies in an ex vivo perfused heart model showed that IL-27 improved cardiac contraction and coronary perfusion, and reduced myocardial injury.

Inflammatory responses in post-ischemic cardiac tissues are often associated with leukocyte accumulation in the myocardium and increased cytokine levels in the blood [6]. IL-6 or IL-6-related cytokines are of particular interest because accumulated evidence suggests that they protect the liver and retina against IR injury [39, 43]. Expression of IL-6 in the myocardium increases after acute myocardial infarction [21]. IL-6 has been demonstrate to be induced in cardiomyocytes after hypoxia/re-oxygenation (which are central to IR-mediated tissue damage) via upregulation of nuclear factor (NF)-κB and NF-IL6 [31, 47]. Moreover, serum IL-6 levels in healthy men increase at 4350 m above sea level, suggesting that hypoxia stimulates IL-6 synthesis [24]. Since circulating levels of IL-27 are also higher among patients with ischemic heart disease [20], we speculated that IL-27 contributes to myocardial repair through its anti-inflammatory effects; it may also trigger pro-survival signals in injured heart tissue. The present study confirmed this notion by showing that expression of IL-27 (both the EBI3 and p28 subunits) in post-ischemic hearts increased at 2–7 days post-IR injury (Fig. 1). EBI3, the larger subunit of IL-27, can either form homodimers or it can form heterodimers with p35 to generate another anti-inflammatory cytokine, IL-35 [3, 4]. Moreover, EBI3 plays an important role in IL-35-mediated regulatory T cell proliferation, a process that modulates chronic inflammatory diseases [7]. Thus, we speculate that EBI3 confers unique cardioprotective properties upon IL-27; however, further studies are needed to clarify this.

Gp130 and WSX-1 comprise the functional IL-27 receptor in immune cells [36]. The results presented herein

ischemic hearts. Exposure of cardiomyocytes isolated from neonatal hearts or cardiomyocyte-like H9c2 cells to SH resulted in cell damage, which was alleviated by prior treatment with either purified or recombinant IL-27 or IL-6. Interestingly, treating cardiomyocytes with IL-27 or IL-6 increased STAT3 activity, signified by an increase in the expression of phosphorylated STAT3. These effects were reversed by a gp130-neutralizing antibody. The gp130-neutralizing antibody not only attenuated STAT3 activity,
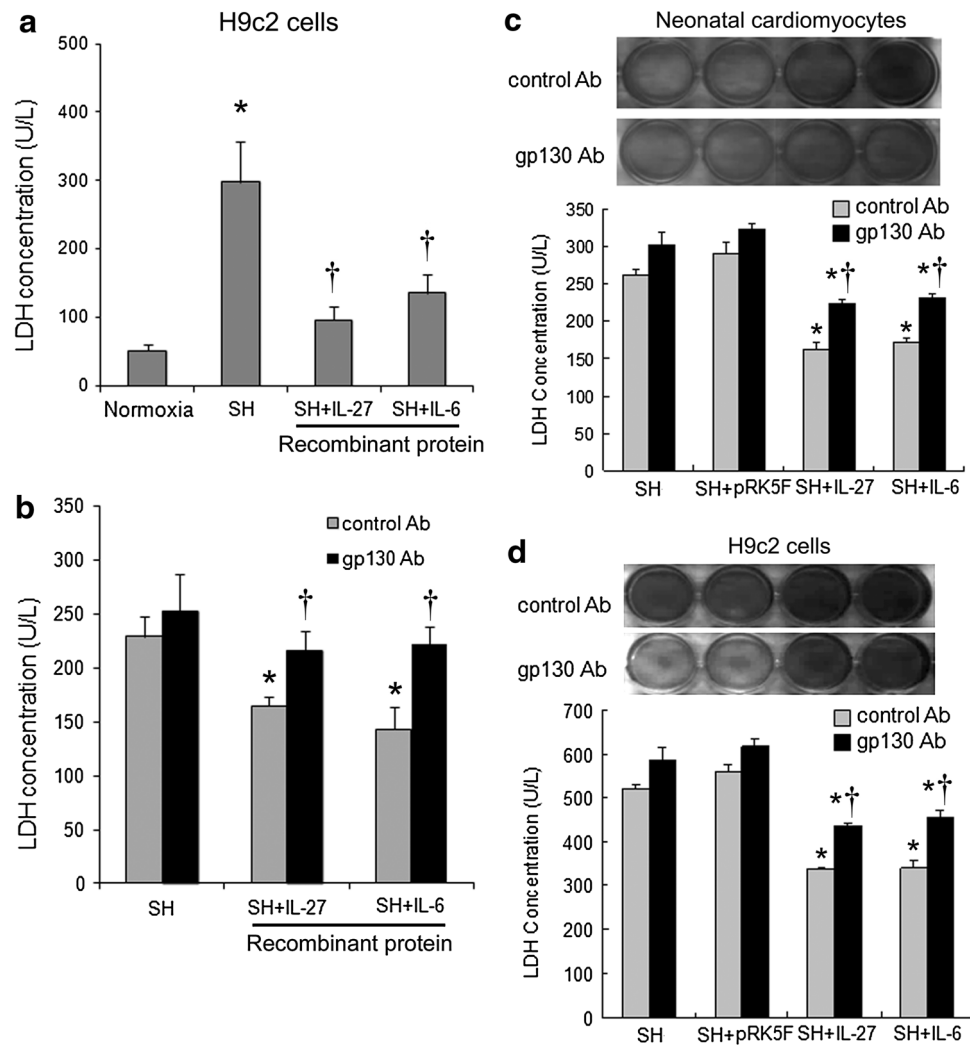
Fig. 5 IL-27-mediated cytoprotection is abolished by treatment with a gp130-neutralizing antibody. **a** H9c2 cells were treated with either recombinant human IL-27 or IL-6 for 6 h prior to the induction of SH (SH, 2 % O$_2$). LDH release was detected after 72 h of SH induction. *$p < 0.05$, SH vs. control cells; †$p < 0.05$, IL-27- or IL-6-treated cells under SH conditions vs. untreated cells under SH conditions. **b** H9c2 cells were pretreated with either recombinant human IL-27 or IL-6 for 6 h in the presence of control or gp130-neutralizing antibodies (5 ng/ml). Cells were then exposed to SH for 72 h and confirm that gp130 and WSX-1 are also expressed in rat cardiac tissues, in isolated neonatal rat cardiomyocytes, and in cardiomyocyte-like H9c2 cells (Fig. 2). This is consistent with a previous study showing that gp130 is widely expressed in many tissues and is important for organ development (including the heart). Similar to IL-1, IL-6, IL-11, leukemia inhibitory factor, and oncostatin M, IL-27 is a cytokine that relies on gp130-associated signal transduction to mediate cellular responses [36]. Cardiac-specific disruption of gp130 results in increased apoptosis of cardiomyocytes in response to ischemia or mechanical stress [18, 46]. By contrast, activation of gp130 promotes

LDH release was measured to access cell viability. *$p < 0.05$, compared with the SH group; †$p < 0.05$, gp130 antibody (Ab) vs. control antibody. **c, d** Neonatal rat cardiomyocytes (**c**) and H9c2 cells (**d**) were pretreated with culture medium containing IL-27 or IL-6 for 6 h in the presence of control or gp130-neutralized antibodies. *Upper panels* show representative culture wells stained with crystal violet (1 %). *Lower panels* show LDH release ($n = 5$). *$p < 0.05$, compared with the SH group treated with the same antibody; †$p < 0.05$, gp130 antibody vs. control antibody

cardiomyocyte survival by inhibiting apoptosis [18, 46]. Therefore, we conclude that cellular signals triggered by the IL-27/gp130/STAT3 pathway play a role in attenuating myocardial cell loss in response to ischemic insult. However, we found that while the expression of gp130 in post-ischemic myocardium decreased, that of WSX-1 did not (Fig. 2). This may mean that gp130-induced pro-survival signals were impaired in the post-ischemic myocardium. Upregulation of IL-27 (EBI3 and p28), as well as unchanged expression of WSX-1, may compensate for reduced levels of gp130 expression to ensure that IL-27 still triggers survival signals in the post-ischemic
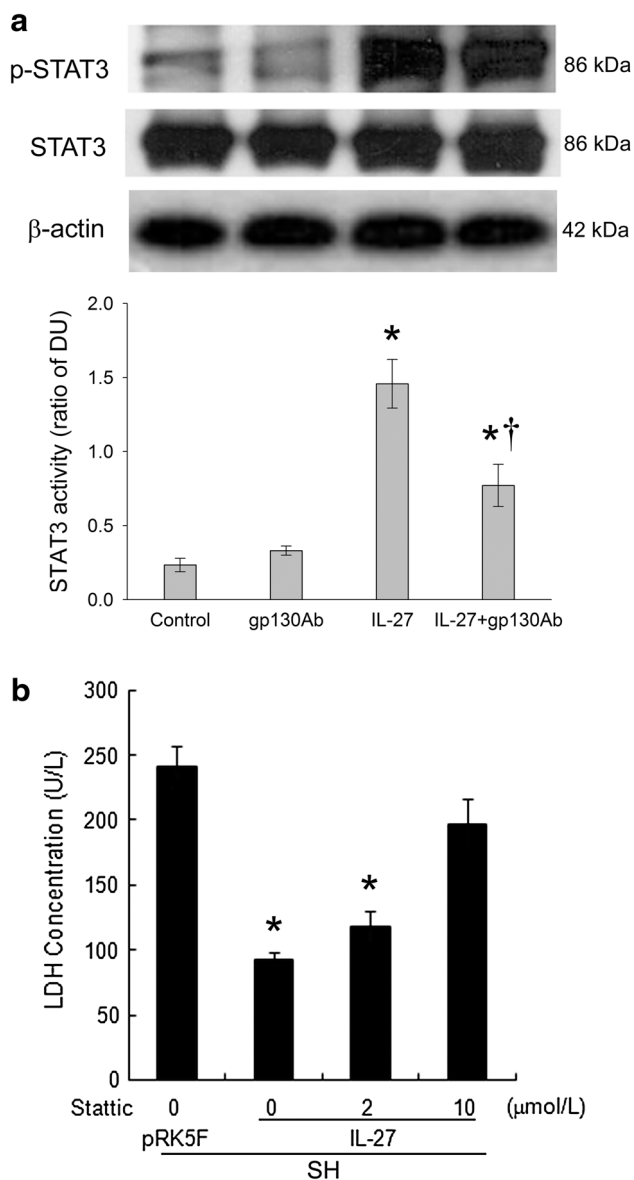
Fig. 6 Gp130 and STAT3 activity are required for IL-27-mediated protection. **a** Representative immunoblots showing expression of phosphorylated STAT3 (p-STAT3), total STAT3, and β-actin in H9c2 cells pretreated with IL-27 for 6 h in the presence or absence of the gp130-neutralizing antibody (5 ng/ml). *Bar graphs* show the STAT3 activity ($n = 3$). *DU* density unit. *$p < 0.05$, compared with the control or gp130 antibody (Ab)-treated group; †$p < 0.05$, IL-27 + gp130 antibody-treated cells vs. IL-27-treated cells. **b** Cells were treated with the STAT3 inhibitor, stattic (0, 2, or 10 μmol/L), for 2 h prior to the addition of IL-27 or pRK5F for 6 h. Cells were then exposed to SH for 72 h. LDH release was measured to evaluate cell viability ($n = 6$). *$p < 0.05$, IL-27- vs. pRK5F-treated cells

Fig. 7 Effects of IL-27 on isolated perfused rat hearts. **a** Changes in coronary perfusion pressure (CPP), left ventricle-developed pressure (LVDP), and coronary flow rate (CF) in rat hearts subjected to 40 min of ischemia followed by 1 h of reperfusion. **b** Hearts were treated with IL-27 or pRK5F (control) 5 min prior to reperfusion ($n = 6$). At the end of the experiment, the perfusate was collected and the levels of the cardiac enzyme, CK-MB (a marker of cardiac injury), were measured. *$p < 0.05$, compared with 0 h; †$p < 0.05$, IL-27-treated vs. pRK5F-treated hearts

myocardium. However, it is not clear why WSX-1 expression is not affected by IR.

STAT plays roles in cell growth, differentiation, and survival [17, 19]. The present study showed that STAT3

activation (as manifest by increased STAT3 phosphorylation) occurs in neonatal cardiomyocytes and H9c2 cells treated with IL-6 or IL-27 (Figs. 3, 4). Moreover, inhibiting STAT3 abrogated the ability of IL-6 and IL-27 to protect cardiac tissue against SH (Fig. 6). This clearly indicates that STAT3 is essential for cardioprotection. Interestingly, other members of the IL-6 family, such as cardiotrophin-1 and leukemia inhibitory factor, also mediate cardiac survival via the STAT3 pathway [26, 33, 38, 42]. Furthermore, cardioprotection mediated by ischemic postconditioning due to a reduction in infarct size is totally abrogated by STAT3 knockout and inhibition [1, 15]. Our observation that cardioprotection is mediated by cytokine-induced STAT3 activation is consistent with that in a previous report showing that IL-10 protects murine hearts against acute myocardial infarction [27]. Subcutaneous administration of recombinant IL-10 (another anti-inflammatory cytokine) increased STAT3 phosphorylation and increased capillary density in the zone bordering the infarct area in murine hearts [27]. Because IL-27 is upregulated in hearts recovering from IR, it will be interesting to find out whether, like IL-10, IL-27 increases angiogenesis in infarct tissues. Vascular endothelial growth factor (VEGF) is an important molecule that participates in repair after hypoxic or ischemic injury due to myocardial infarction by improving perfusion of the peri-infarct and infarct areas [29]. Like IL-27, oncostatin M belongs to the gp130 family and is expressed in human atherosclerotic lesions; it stimulates the production of VEGF, which contributes to plaque angiogenesis [9]. The results presented herein show that IL-27 improves coronary perfusion during recovery from IR (Fig. 7a). Though the present study did not explore how actors downstream of the IL-27/gp130/STAT3 pathway may affect VEGF expression, a previous study shows that inhibiting STAT3 abrogates gp130-induced VEGF synthesis in smooth muscle cell cultures [9]. This indicates that the IL-27/gp130/STAT3 pathway may enhance the function of VEGF during coronary perfusion. We previously demonstrated that VEGF levels in rat myocardium increased after exposure to chronic hypoxia and that VEGF plays a role in cardiac tolerance to coronary ligation-induced IR injury [29]. Further studies are warranted to examine whether the effects of IL-27 on cardioprotection are dependent upon VEGF.

Redox imbalance-induced oxidative stress contributes to myocardial injury in post-ischemic hearts [34]. Therefore, it would be interesting to see whether the cardioprotective effects of the IL-27/STAT3 pathway are, at least in part, due to the scavenging of reactive oxygen species and improved antioxidant defense. Though the present study did not explore this possibility, previous studies show that IL-6, as well as other cytokines such as TNF-α, increase the expression of manganese superoxide dismutase (MnSOD), an endogenous antioxidant protein, in IR tissues [11, 44]. Moreover, IL-6-induced STAT3 activation increases MnSOD activity [33]. Loss of STAT3 in a genetic knockout mouse model showing reduced MnSOD levels had a negative effect on cardioprotection and increased infarct size after IR [33]. As well as being expressed in the nucleus, STAT3 is also expressed in the mitochondria of cardiac cells [45]. The cellular effects of mitochondrial dysfunction, such as impaired cell respiration for ATP synthesis, increased free radical generation, and the release of pro-apoptotic molecules, play a role in IR-induced cardiomyocyte death. Therefore, the cardioprotective effects of IL-27 may be based on downstream effects of STAT3, including the regulation of mitochondrial function and the activation of SAFE pathways [1, 28]. Moreover, previous findings in chick embryonic hearts reveal that protein kinases (including Akt, extracellular signal-regulated kinase 2, and glycogen synthase kinase 3β) within the Reperfusion Injury Salvage Kinase (RISK) pathway are activated downstream of STAT3 [35]. Therefore, further studies should examine whether the RISK pathway participates in IL-27/STAT3 signaling to protect against IR-mediated cardiac injury.

We conclude that IL-27 plays a novel role of cardioprotection during myocardial recovery from IR insult. IL-27 attenuates myocardial damage by activating gp130/STAT3, and protects cardiomyocytes from SH or IR. These results indicate that the induction of pro-survival signals via IL-27/gp130/STAT3 may be a potential therapeutic strategy for the treatment of ischemic myocardial injury.

References

1. Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, Schulz R (2008) Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. Circ Res 102:131–135. doi:10.1161/CIRCRESAHA.107.164699
2. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R (2008) The myocardial JAK/STAT pathway: from protection to failure. Pharmacol Ther 120:172–185. doi:10.1016/j.pharmthera.2008.08.002
3. Brombacher F, Kastelein RA, Alber G (2003) Novel IL-12 family members shed light on the orchestration of Th1 responses. Trends Immunol 24:207–212. doi:10.1016/S1471-4906(03)00067-X
4. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA (2011) Cutting edge: human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. J Immunol 186:6661–6666. doi:10.4049/jimmunol.1100315
5. Chen JC, Huang AJ, Chen SC, Wu CL, Wu WM, Chiang HS, Chan CH, Lin CM, Huang YT (2012) Interleukin-27 and

interleukin-12 augment activation of distinct cord blood natural killer cells responses via STAT3 pathways. J Formos Med Assoc 111:275–283. doi:10.1016/j.jfma.2010.10.002

6. Cheng X, Liao YH, Ge H, Li B, Zhang J, Yuan J, Wang M, Liu Y, Guo Z, Chen J, Zhang L (2005) TH1/TH2 functional imbalance after acute myocardial infarction: coronary arterial inflammation or myocardial inflammation. J Clin Immunol 25:246–253. doi:10.1007/s10875-005-4088-0

7. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA (2007) The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 450:566–569. doi:10.1038/nature06306

8. Dawn B, Xuan YT, Guo Y, Rezazadeh A, Stein AB, Hunt G, Wu WJ, Tan W, Bolli R (2004) IL-6 plays an obligatory role in late preconditioning via JAK-STAT signaling and upregulation of iNOS and COX-2. Cardiovasc Res 64:61–71. doi:10.1016/j.cardiores.2004.05.011

9. Demyanets S, Kaun C, Rychli K, Pfaffenberger S, Kastl SP, Hohensinner PJ, Rega G, Katsaros KM, Afonyushkin T, Bochkov VN, Paireder M, Huk I, Maurer G, Huber K, Wojta J (2011) Oncostatin M-enhanced vascular endothelial growth factor expression in human vascular smooth muscle cells involves PI3K-, p38 MAPK-, Erk1/2- and STAT1/STAT3-dependent pathways and is attenuated by interferon-gamma. Basic Res Cardiol 106:217–231. doi:10.1007/s00395-010-0141-0

10. Devergne O, Coulomb-L'Hermine A, Capel F, Moussa M, Capron F (2001) Expression of Epstein-Barr virus-induced gene 3, an interleukin-12 p40-related molecule, throughout human pregnancy: involvement of syncytiotrophoblasts and extravillous trophoblasts. Am J Pathol 159:1763–1776. doi:10.1016/S0002-9440(10)63023-4

11. Dougall WC, Nick HS (1991) Manganese superoxide dismutase: a hepatic acute phase protein regulated by interleukin-6 and glucocorticoids. Endocrinology 129:2376–2384. doi:10.1210/endo-129-5-2376

12. Frangogiannis HG, Smith CW, Entman ML (2002) The inflammatory response in myocardial infarction. Cardiovasc Res 53:31–47. doi:10.1016/S0008-6363(01)00434-5

13. Fuglesteg BN, Suleman N, Tiron C, Kanhema T, Lacerda L, Andreasen TV, Sack MN, Jonassen AK, Mjos OD, Opie LH, Lecour S (2008) Signal transducer and activator of transcription 3 is involved in the cardioprotective signalling pathway activated by insulin therapy at reperfusion. Basic Res Cardiol 103:444–453. doi:10.1007/s00395-008-0728-x

14. Heusch G, Boengler K, Schulz R (2008) Cardioprotection: nitric oxide, protein kinases, and mitochondria. Circulation 118:1915–1919. doi:10.1161/CIRCULATIONAHA.108.805242

15. Heusch G, Musiolik J, Gedik N, Skyschally A (2011) Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. Circ Res 109:1302–1308. doi:10.1161/CIRCRESAHA.111.255604

16. Hilfiker-Kleiner D, Kaminski K, Podewski E, Bonda T, Schaefer A, Sliwa K, Forster O, Quint A, Landmesser U, Doerries C, Luchtefeld M, Poli V, Schneider MD, Balligand JL, Desjardins F, Ansari A, Struman I, Nguyen NQ, Zschemisch NH, Klein G, Heusch G, Schulz R, Hilfiker A, Drexler H (2007) A cathepsin D-cleaved 16 kDa form of prolactin mediates postpartum cardiomyopathy. Cell 128:589–600. doi:10.1016/j.cell.2006.12.036

17. Hirano T, Ishihara K, Hibi M (2000) Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19:2548–2556. doi:10.1038/sj.onc.1203551

18. Hirota H, Chen J, Betz UA, Rajewsky K, Gu Y, Ross J Jr, Muller W, Chien KR (1999) Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. Cell 97:189–198. doi:10.1016/S0092-8674(00)80729-1

19. Ihle JN (1996) STATs: signal transducers and activators of transcription. Cell 84:331–334

20. Jafarzadeh A, Nemati M, Rezayati MT (2011) Serum levels of interleukin (IL)-27 in patients with ischemic heart disease. Cytokine 56:153–156. doi:10.1016/j.cyto.2011.06.014

21. Kaneko K, Kanda T, Yokoyama T, Nakazato Y, Iwasaki T, Kobayashi I, Nagai R (1997) Expression of interleukin-6 in the ventricles and coronary arteries of patients with myocardial infarction. Res Commun Mol Pathol Pharmacol 97:3–12

22. Kelly RF, Lamont KT, Somers S, Hacking D, Lacerda L, Thomas P, Opie LH, Lecour S (2010) Ethanolamine is a novel STAT-3 dependent cardioprotective agent. Basic Res Cardiol 105:763–770. doi:10.1007/s00395-010-0125-0

23. Kimes BW, Brandt BL (1976) Properties of a clonal muscle cell line from rat heart. Exp Cell Res 98:367–381. doi:10.1016/0014-4827(76)90447-X

24. Klausen TON, Poulsen TD, Richalet JP, Pedersen BK (1997) Hypoxemia increases serum interleukin-6 in humans. Eur J Appl Physiol Occup Physiol 76:480–482

25. Kleinbongard P, Heusch G, Schulz R (2010) TNFα in atherosclerosis, myocardial ischemia/reperfusion and heart failure. Pharmacol Ther 127:295–314. doi:10.1016/j.pharmthera.2010.05.002

26. Kodama H, Fukuda K, Pan J, Makino S, Baba A, Hori S, Ogawa S (1997) Leukemia inhibitory factor, a potent cardiac hypertrophic cytokine, activates the JAK/STAT pathway in rat cardiomyocytes. Circ Res 81:656–663. doi:10.1161/01.RES.81.5.656

27. Krishnamurthy P, Rajasingh J, Lambers E, Qin G, Losordo DW, Kishore R (2009) IL-10 inhibits inflammation and attenuates left ventricular remodeling after myocardial infarction via activation of STAT3 and suppression of HuR. Circ Res 104:e9–e18. doi:10.1161/CIRCRESAHA.108.188243

28. Lacerda L, Somers S, Opie LH, Lecour S (2009) Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. Cardiovasc Res 84:201–208. doi:10.1093/cvr/cvp274

29. Lin JS, Chen YS, Chiang HS, Ma MC (2008) Hypoxic preconditioning protects rat hearts against ischaemia-reperfusion injury: role of erythropoietin on progenitor cell mobilization. J Physiol 586:5757–5769. doi:10.1113/jphysiol.2008.160887

30. Lu MJ, Chen YS, Huang HS, Ma MC (2014) Hypoxic preconditioning protects rat hearts against ischemia–reperfusion injury via the arachidonate12-lipoxygenase/transient receptor potential vanilloid 1 pathway. Basic Res Cardiol 109:414. doi:10.1007/s00395-014-0414-0

31. Matsui H, Ihara Y, Fujio Y, Kunisada K, Akira S, Kishimoto T, Yamauchi-Takihara K (1999) Induction of interleukin (IL)-6 by hypoxia is mediated by nuclear factor (NF)-kB and NF-IL6 in cardiac myocytes. Cardiovasc Res 42:104–112. doi:10.1016/S0008-6363(98)00285-5

32. Matsushita K, Oda T, Kimura K, Shimada M, Sano M, Umezawa A, Hata J, Ogawa S (2005) Interleukin-6/soluble interleukin-6 receptor complex reduces infarct size via inhibiting myocardial apoptosis. Lab Invest 85:1210–1223. doi:10.1038/labinvest.3700322

33. Negoro S, Kunisada K, Fujio Y, Funamoto M, Darville MI, Eizirik DL, Osugi T, Izumi M, Oshima Y, Nakaoka Y, Hirota H, Kishimoto T, Yamauchi-Takihara K (2001) Activation of signal transducer and activator of transcription 3 protects cardiomyocytes from hypoxia/reoxygenation-induced oxidative stress through the upregulation of manganese superoxide dismutase. Circulation 104:979–981. doi:10.1161/hc3401.095947

34. Pagliaro P, Penna C (2014) Redox signaling and cardioprotection—translatability and mechanism. Br J Pharmacol. doi:10.1111/bph.12975

35. Pedretti S, Raddatz E (2011) STAT3alpha interacts with nuclear GSK3beta and cytoplasmic RISK pathway and stabilizes rhythm in the anoxic-reoxygenated embryonic heart. Basic Res Cardiol 106:355–369. doi:10.1007/s00395-011-0152-5

36. Pflanz S, Hibbert L, Mattson J, Rosales R, Vaisberg E, Bazan JF, Phillips JH, McClanahan TK, de Waal Malefyt R, Kastelein RA (2004) WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. J Immunol 172:2225–2231. doi:10.4049/jimmunol.172.4.2225

37. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, Hibbert L, Churakova T, Travis M, Vaisberg E, Blumenschein WM, Mattson JD, Wagner JL, To W, Zurawski S, McClanahan TK, Gorman DM, Bazan JF, de Waal Malefyt R, Rennick D, Kastelein RA (2002) IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 16:779–790

38. Railson JE, Liao Z, Brar BK, Buddle JC, Pennica D, Stephanou A, Latchman DS (2002) Cardiotrophin-1 and urocortin cause protection by the same pathway and hypertrophy via distinct pathways in cardiac myocytes. Cytokine 17:243–253. doi:10.1006/cyto.2001.1011

39. Sanchez RN, Chan CK, Garg S, Kwong JM, Wong MJ, Sadun AA, Lam TT (2003) Interleukin-6 in retinal ischemia reperfusion injury in rats. Invest Ophthalmol Vis Sci 44:4006–4011. doi:10.1159/000050880

40. Shyu KG, Chen CC, Wang BW, Kuan P (2001) Angiotensin II receptor antagonist blocks the expression of connexin43 induced by cyclical mechanical stretch in cultured neonatal rat cardiac myocytes. J Mol Cell Cardiol 33:691–698. doi:10.1006/jmcc.2000.1333

41. Smart N, Mojet MH, Latchman DS, Marber MS, Duchen MR, Heads RJ (2006) IL-6 induces PI3-kinase and nitric oxide-dependent protection and preserves mitochondrial function in cardiomyocytes. Cardiovasc Res 69:164–177. doi:10.1016/j.cardiores.2005.08.017

42. Takahashi N, Saito Y, Kuwahara K, Harada M, Tanimoto K, Nakagawa Y, Kawakami R, Nakanishi M, Yasuno S, Usami S, Yoshimura A, Nakao K (2005) Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes. J Mol Cell Cardiol 38:185–192. doi:10.1016/j.yjmcc.2004.10.016

43. Tiberio L, Tiberio GA, Bardella L, Cervi E, Cerea K, Dreano M, Garotta G, Fra A, Montani N, Ferrari-Bravo A, Callea F, Grigolato P, Giulini SM, Schiaffonati L (2006) Mechanisms of interleukin-6 protection against ischemia–reperfusion injury in rat liver. Cytokine 34:131–142. doi:10.1016/j.cyto.2006.04.009

44. Tsan MF, White JE, Treanor C, Shaffer JB (1990) Molecular basis for tumor necrosis factor-induced increase in pulmonary superoxide dismutase activities. Am J Physiol 259:L506–L512. doi:10.1016/S1074-7613(02)00324-2

45. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, Larner AC (2009) Function of mitochondrial Stat3 in cellular respiration. Science 323:793–797. doi:10.1126/science.1164551

46. Yamauchi-Takihara K, Kishimoto T (2000) Cytokines and their receptors in cardiovascular diseases–role of gp130 signalling pathway in cardiac myocyte growth and maintenance. Int J Exp Pathol 81:1–16. doi:10.1046/j.1365-2613.2000.00139.x

47. Yan SF, Zou YS, Mendelsohn M, Gao Y, Naka Y, Du Yan S, Pinsky D, Stern D (1997) Nuclear factor interleukin 6 motifs mediate tissue-specific gene transcription in hypoxia. J Biol Chem 272:4287–4294. doi:10.1074/jbc.272.7.4287

48. Yoshida H, Miyazaki Y (2008) Regulation of immune responses by interleukin-27. Immunol Rev 226:234–247. doi:10.1111/j.1600-065X.2008.00710.x